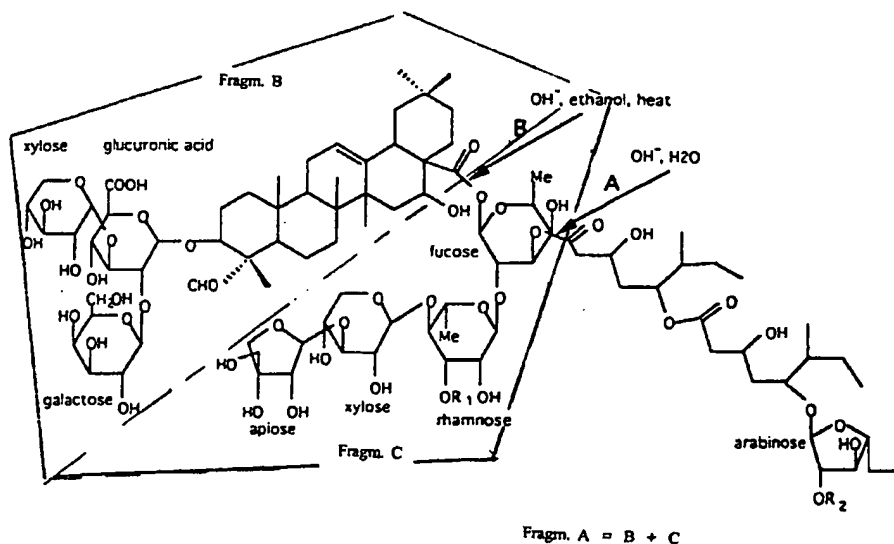




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(54) Title: COMPOUNDS WITH ADJUVANT ACTIVITY



(57) Abstract

The present invention relates to a plant cell extract with adjuvant activity obtainable by extracting fresh plant material consisting of substantially living cells, originating from saponin producing plants of the genera *Quillaja*, *Saponaria* or *Gypsophila*. Furthermore the invention provides compounds having adjuvant activity obtainable by separating the plant cell extract into its components. Both the extract and the compounds thus obtained have various applications in adjuvants and vaccines.

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COMPOUNDS WITH ADJUVANT ACTIVITY

The present invention relates to compounds with adjuvant activity, in particular saponins. The invention relates to compounds in very pure form, hydrolyzed derivatives of these pure compounds and modified derivatives thereof. The invention further relates to methods for manufacturing the compounds, the use of the compounds in ISCOM matrices, ISCOMS, adjuvants and vaccines, and the ISCOM matrices, ISCOMS, adjuvants and vaccines as such which contain the compounds, and to a method for controlling the immune response in addition to the targeted release of medicines. The compounds can also be used for precise and selective hemolysis of erythrocytes.

Saponins are substances which occur inter alia in plants of for instance the genera Saponaria, Gypsophyllia and in particular Quillaja. Although the chemical structure of these saponins has not been fully clarified, they contain in any case gypsogenin or the gypsogenenic acid respectively Quillajic acid derived therefrom, all triterpenoids and a number of sugar chains. In many cases there also occurs a fatty acid residue which is linked to a sugar residue on the triterpenoid structure.

Of saponins it is known that they exhibit adjuvant activity. They are therefore regularly used as adjuvants in vaccines against for instance foot-and-mouth disease. It has been found that the adjuvant activity is related to particular components of the crude extract of plants, in particular those of the genus Quillaja.

It has already been demonstrated that a component obtained after purifying a dialyzed extract of the bark of Quillaja saponaria Molina on an ion exchanger column of the DE-52 type followed by Sephadex G50 gel filtration produces a fraction which is called QUIL A. This QUIL A exhibits a strong adjuvant activity (Dalsgaard, K., Arch. ges. Virusforsch. 44, 243-254 (1974)). QUIL A itself is however still not a pure substance, as has been found after separation on "reverse phase" HPLC (RP-HPLC). The QUIL A is

found to consist of a large number of components. Nor does the use of a dialyzed extract soluble in methanol produce any better result and is found to be still very heterogeneous, even when fractions thereof are further
5 purified (WO 88/09336).

The drawback to such a non-pure substance is that both the activity and the side-effects, such as irritation, allergy and the like, of the substance cannot be predicted and controlled. Each QUIL A fraction from each extract will
10 have a different composition and will have to be tested, for instance for activity, before use.

There therefore exists a need to obtain the components from the QUIL A or from other saponin-containing extracts or fractions thereof in a very pure form in order to enable
15 better control of the activity and the like. This is particularly important when there is a desire to use the saponins as adjuvants in vaccines for human use. Strict requirements are made of such adjuvants in respect of purity.

20 It has now been found that a plant extract obtainable by extraction of selected fresh plant material consisting of substantially living cells is much less heterogeneous than the starting materials used heretofore for the production of QUIL A and the compounds isolated therefrom. It appeared
25 that this fresh material comprises essentially two main components, both having adjuvant activity. This fresh material obtained in a similar manner as known QUIL A thus consists of less components and has a lower toxicity and a similar or higher adjuvant activity than QUIL A.
30 Furthermore, from this material relatively high amounts of the two single main components may be purified by means of purification techniques known per se, such as reversed phase HPLC, because of its simple composition as compared to QUIL A. The invention therefore provides a plant cell extract,
35 which is a more pure starting material, and in itself already suitable as an adjuvant. The invention further relates to compounds obtainable by separating into fractions an extract of fresh plant material consisting of

substantially living cells which originates from saponin-containing plants.

That the compounds according to the invention can be obtained in very pure form can probably be explained by the fact that in fresh plant material consisting of substantially living cells no oxidation or decomposition of the compounds present therein has yet occurred. Heretofore it was always assumed that only the use of the bark of 50-100 year-old trees could provide sufficient saponins. The use of young material was not therefore considered. It has been found however that generally more heterogenous mixtures of saponins are obtained from the bark of old trees. Added to this is the fact that in current practice the bark, after being removed from the trees, is exposed for a long time to the weather conditions, whereby, as has now been found, the active compounds can be decomposed or can oxidize. An additional drawback to the use of the bark of old trees is that there is a danger of a shortage of old trees (Hoffman, J., A.E., Flores Silvestre de Chile, p. 102, Ediciones Fundacion Claudio Goy (1991)).

In this description understood by the term "plant cell extract" is an extract obtainable by extracting plant material, such as single cells but also complete small trees or parts thereof, such as roots, cambium, phloem etc., with water. In the remainder of the text the term plant cell extract will be used in addition to "New Quil A" in order to differentiate the new extract according to the invention from known heterogenous QUIL A.

Understood in this description by the term "saponins" are those compounds which can be obtained directly from a plant extract and do not undergo any subsequent hydrolysis or modification treatment. "Saponin-like compounds" are compounds which do undergo such a hydrolysis or modification. The general term "compounds" includes both de saponins and the saponin-like compounds.

When "fresh plant material" is mentioned in the text, this means plant material extracted essentially "directly after harvesting" without subjecting the material to weather

conditions, such that conditions stimulating oxidation and decomposition can occur as less as possible. Furthermore it appeared that plant material isolated from parts of a plant which are not differentiated or have been differentiated a short time before, such as the cambium, young wood and phloem as well as the roots, comprise less decomposition products derived from saponin.

The term "ISCOM matrix" represents a matrix for immunostimulating complexes as described in WO 90/03184.

By "ISCOMS" is meant an ISCOM matrix having enclosed therein at least one antigen, as described in US patent 4,578,269.

The sources from which the saponin-like compounds according to the present invention can be recovered are very diverse. An important condition however is that use is made of fresh plant material consisting of substantially living cells. It is also important that the material is processed immediately after harvesting thereof. Suitable sources are for instance the trunks of trees up to 15 years old of the type Quillaja saponaria and the roots of such trees. In principle the layer of living cells of older trees consisting of growing wood, cambium and phloem and the roots could also be used, but this is less desirable with regard to the scarcity of older trees. In addition tissue cultures and suspension cell cultures of cells originating particularly from the cambium are also very suitable sources, as described in the Netherlands patent application 93.01404, the content of which is incorporated herein by way of reference, as well as root cultures and the like.

After separation on a RP-HPLC column two different compounds are found, which are called QUADRI-1 and QUADRI-2. From analytical RP-HPLC it is found that very pure substances of both QUADRI-1 and QUADRI-2 99.99% pure material can be obtained in this way.

The invention further relates to the hydrolysis products derived from the saponins. It has been found that by selective hydrolysis of the pure saponins the adjuvant activity can be varied. Different compounds have been found

by means of different methods of alkaline hydrolysis. On the basis of the hydrophobicity of the products and assuming the structure as is proposed by Higuchi et al. (in Phytochemistry 26, 229-235 (1987)) and shown in figure 6, the acyl group with the arabino sugar residue is first cleaved to obtain product A. Starting from QUADRI-1 respectively QUADRI-2 these compounds are characterized by their comparatively strong hydrophilicity characterized by a retention time of about 7.968 minutes (in figure 7; 8.645 in figure 10), respectively about 9.6 minutes (in figure 8; 10.36 in figure 10).

These compounds are further characterized by their sugar content as shown in table 2. Subsequently, after more intensive hydrolysis, the fucose with sugars linked thereto are also cleaved, which results in compounds QUADRI-1B and QUADRI-2B which are found to be identical, both with respect to their NMR spectra and with respect to the point of time they leave the column at about 13.1 (figure 8 and figure 10) (see also table 2).

The compounds according to the invention, and then particularly the hydrolyzed derivatives thereof, can also serve as basis for modified compounds. It is thus for instance possible to link a peptide to the acid group in position 23 of product B, respectively the fucose of product A. The compound modified with the peptide can itself or in combination with other saponins or saponin-like compounds be used as adjuvant and also in the formation of ISCOMs.

Another possible application of the compound according to the invention is the targeted release of medicines. Particularly the hydrolyzed compounds could be useful for this purpose.

The saponins according to the present invention can be prepared by means of a per se known method. Such a method is for instance described by Dalgaard (supra). Separation into fractions of the product ("New Quil A") obtained by means of this method can take place using preparative HPLC, as for instance RP-HPLC, e.g. on a Vydac C4-column with a gradient

of 30-45% acetonitril with 0.15 % TFA. Any separation of an extract into its separate components can however be used in the preparation of the compounds according to the invention.

The compounds according to the invention exhibit
5 adjuvant activity to a lesser or greater extent. They can be used alone or in combination, for instance in vaccines. It is possible to manufacture an ISCOM matrix from compounds according to the invention or combinations thereof together with for example cholesterol and phosphatidylcholin. The
10 inclusion of one or more antigens in the matrix provides ISCOMs.

The present invention is illustrated in this description with reference to saponins and saponin-like compounds of Quillaja saponaria, but is not limited thereto.

15 Of a number of ISCOMS it has been demonstrated that, in addition to the MHC-class 2 response usual for normal adjuvants which results in increased T-helper cell concentration, they can also induce a heretofore unique MHC-class 1 response. It has been further demonstrated that the
20 induced T-helper cells are mostly of the TH-1 type and thus give protection and are to a lesser extent or not at all of the TH-2 type which result in an over-sensitivity reaction.

QUADRI-1 and QUADRI-2 per se and as part of ISCOMS possess such an adjuvant function and can thus also induce
25 cytolytic T-lymphocytes (CTLs). The hydrolyzed compounds also exhibit these properties but not to the same extent. They possibly help to dissolve the antigens and appear to induce a lower MHC-class 2 immune response.

Because in the studies leading to the invention a much
30 clearer insight has been gained into how the diverse components forming part of the crude extract occur, particularly due to partial hydrolysis of QUADRI-1 and QUADRI-2, there is a much better understanding of the origin of the differences in adjuvant activity. QUADRI-1 and
35 QUADRI-2 are surrounded by many more sugar residues than the derived products QUADRI-1A, -1B, -1D and QUADRI-2A, -2B, -2D generated by selective hydrolysis, which will influence among other things the hydrophobicity and the three-

dimensional structure. The presentation of antigens can hereby be selectively influenced.

Saponins can also be used for the hemolysis of erythrocytes. A known method wherein use is made of hemolysis is the counting of white blood cells using a Coulter counter. Normally speaking, the accuracy of the count is influenced highly negatively by the presence of the red blood cells. By lysing the red blood cells the disruptive background is removed. Saponins can be used to lyse erythrocytes. It has been found that the existing saponin preparations are not reliable enough to standardize this method of counting white blood cells. There is a demand from both industry and the legislator for a pure preparation with which this white blood cell determination can be performed in reproducible manner. The saponins, and optionally saponin-like compounds, according to the invention meet this requirement. In addition it has been found that different types of erythrocytes, such as for instance normal erythrocytes or sickle cells, react differently to the saponin preparations according to the invention. That is, that for hemolysis of the different types of erythrocytes different concentrations of the saponins are necessary. The specificity of the pure saponins, respectively the mixture of QUADRI-1 and QUADRI-2 prior to RP-HPLC thereof, provides wholly new application possibilities in the field of hemolysis.

Another field of application of the compounds according to the invention is the targeted release of medicines. Herein a quantity of medicine is included in an ISCOM matrix. By means of molecules with a targeted function, such as for instance antibodies, which are for example included in the matrix, the ISCOMS can be transported to the desired location in the body.

The invention will be further elucidated with reference to a number of examples, which are not however intended to limit the invention in any way.

EXAMPLE 1

Purification and analysis of saponins according to the invention.

In accordance with the Dalsgaard method (supra) "New
5 Quil A" is recovered from plant cell material freshly
extracted from approximately 15 year-old Quillaja trees. The
Dalsgaard method consists essentially of a purifying of a
dialyzed extract on an ion exchanger column of the type DE-
52 followed by Sephadex G50 gel filtration. It is also
10 possible to use ultrafiltration instead of gel filtration.

The thus obtained "New Quil A" is subjected to a RP-
HPLC (VYDAC®, C₄-column, eluted with 30%-45% acetonitrile
in a 0.15% aqueous TFA-solution). The HPLC elution pattern
shows two main peaks which are called respectively QUADRI-1
15 and QUADRI-2 (see figure 1).

The same procedure is performed on plant cell material
obtained by means of tissue culture or suspension cell
culture. Figure 2 shows the RP-HPLC elution pattern of QUIL
A that is isolated from a suspension cell culture. In
20 addition to the two peaks characteristic of QUADRI-1 and
QUADRI-2 a number of peaks can also be distinguished which
were later shown to be deacylated versions of QUADRI-2.

By way of comparison the HPLC pattern of QUIL A from
old bark is shown in figure 3. The arrows in figure 3
25 indicate the positions of QUADRI-1 and QUADRI-2 and of
deacylated QUADRI-2. It can be seen from this figure that
the material from the old bark is not just much more
heterogenous than the material recovered according to the
invention from young trees, but also shows double peaks
30 where in fig. 1 single peaks may be seen.

By way of example in figure 11 is shown the HPLC
elution pattern of a component known under the name B4b. It
is known of B4b that it has a very high ISCOM forming
capability but a low adjuvant activity (WO 90/03184). The
35 QUADRI-1 and QUADRI-2 peaks are absent from the HPLC pattern
and it is precisely the deacylated QUADRI-1A and QUADRI-2A
which are present. From this it appears that fraction B4b
comprises various components upon analysis on RP-HPLC. The

absence of QUADRI-1 and QUADRI-2 can now also explain why B4b does provide ISCOMS but has no adjuvant activity normal for ISCOMS.

Figures 4 and 5 show RP-HPLC elution patterns for the compounds QUADRI-1 and QUADRI-2 respectively. It can be seen from the figures that both fractions are more than 99.99% pure.

EXAMPLE 2

Identification of QUADRI-1 and QUADRI-2

Different techniques were used to solve the structure of QUADRI-2 in particular and compare it where necessary with QUADRI-1. FAB-mass spectrometry leads unambiguously to a molecular mass of 1988 Dalton ($M+H=1989$ in fig. 12) for QUADRI-2 and 2150 Dalton for QUADRI-1. Both mass spectra of QUADRI-1 and QUADRI-2 show a clear peak at a mass which is 132 lower than the main peak. This peak represents a fragment ion, in which one pentose is missing as compared to the starting compound. Theoretically such an ion may also indicate that the main product is contaminated with a by-product, in which the said pentose is missing by nature.

Shown in the tables 1 and 2 is the composition of the sugars present, which is determined according to the method of Kamerling, J.P. et al., Carbohydrates ..., 175-263, (1989), A.M. Lawson (ed.), Clin. Biochem. 1, Mass Spectrometry, W. de Gruyter, New York. Of QUADRI-1 0.4 mg was used with 250 nmol mannitol as Internal Standard (IS). The results are shown in the first part of table 1. Of QUADRI-2 0.3 mg was used with 250 nmol IS. The results are shown in the second part of table 1. The relative amount of sugars was also determined for hydrolysis fragments (A and B, compare figs. 7 and 8) of QUADRI-1 and -2, obtained by means of the alkaline hydrolysis described in example 4. The results are listed in Table 2. Of QUADRI-1A 250 μ l solution was used with 20 nmol IS. The results are shown in the first part of table 2. Of QUADRI-1B 250 μ l was likewise used with 20 nmol IS. The results of the hydrolysates of QUADRI-2

obtained in a similar manner are shown in the second part of table 2.

It can be seen from table 1 that QUADRI-1 contains 415.9 μg sugars per mg. This corresponds with 41.6% carbohydrates. QUADRI-2 is less glycosylated and contains 359.6 μg per mg (35.9% carbohydrates).

TABLE 1

	Monosaccharide	nmol/mg	mol.ratio	$\mu\text{g}/\text{mg}$
10	QUADRI-1			
	Apiose*	250.0	0.8	37.5
	Arabinose	285.0	1.0	42.8
	Rhamnose	290.0	1.0	47.6
15	Fucose	280.0	0.9	45.9
	Xylose	550.0	1.9	82.5
	Glucuronic acid	281.3	1.0	54.6
	Galactose	295.6	1.0**	53.2
	Glucose	287.5	1.0	51.8
20	-----			
	Monosaccharide	nmol/mg	mol.ratio	$\mu\text{g}/\text{mg}$
	QUADRI-2			
	Apiose*	233.3	0.8	35.0
25	Arabinose	285.0	1.0	42.8
	Rhamnose	280.0	1.0	45.9
	Fucose	280.0	1.0	45.9
	Xylose	546.6	1.9	82.0
	Glucuronic acid	270.0	1.0	52.4
30	Galactose	284.2	1.0**	51.1
	Glucose	25.0	0.09	4.5

* For this sugar no reference is included. It is assumed that the remaining peak pattern represents apiose. MS analysis indicates a pentose but in view of the GC retention time it is not a known linear pentose.

** The quantity of this sugar is set at 1 in the molecular ratio.

TABLE 2

5	molecular ratio				
	mild alkaline hydrolysis		strong alkaline hxdrolysis		
10	peak 1 QUADRI-1A	peak 1 void vol.	peak 2 QUADRI-1A	peak 3 QUADRI-1B	
	Apiose*	0.9	0.8	0.9	-
	Arabinose	0.2	0.1	-	-
15	Rhamnose	1.0**	1.0**	1.0**	-
	Fucose	1.0	0.8	1.0	-
	Xylose	1.9	1.0	1.9	1.0
	Glucuronic acid	0.8	-	1.0	1.0
	Galactose	0.8	-	0.9	1.0**
20	Glucose	0.8	0.9	1.0	0.05
	peak 1 QUADRI-2A	peak 1 void vol.	peak 2 QUADRI-2A	peak 3 QUADRI-2B	
25	Apiose*	0.7	n.d.	0.8	-
	Arabinose	-	n.d.	-	-
	Rhamnose	1.0	n.d.	1.0	-
	Fucose	1.0	n.d.	1.0	-
	Xylose	2.0	n.d.	1.9	0.8
30	Glucuronic acid	1.3	n.d.	0.9	1.0
	Galactose	1.2	n.d.	0.9	0.9
	Glucose	-	n.d.	-	-

The significance of * and ** is as given in table 1.

35 n.d. = not done

Comparison of tables 1 and 2, in terms of comparing QUADRI-1 and 2 with the fragments 1A and 2A respectively, shows that only arabinose is absent and thereby proves that the arabinose, and therewith very probably also the fatty acid part, is only cleaved after alkaline hydrolysis. With strongly alkaline hydrolysis the fucose and the sugar chain linked thereto is also found to be cleaved, as is shown from the void volume peak which, in the case of QUADRI-1, contains precisely those sugars which are suspected in this side chain. The three remaining sugars on the other side of the Quillajic acid are also not hydrolyzed during strong hydrolysis and thus form fragment QUADRI-1B respectively QUADRI-2B.

Besides with FAB mass spectrometry and sugar analysis, QUADRI-2 in particular was characterized still further making use of GC-MS, NMR (500 MHz), UV/vis-spectra, Fluorescence and Circular Dichroism. In GC-MS sugar analysis, in addition to the sugars, Quillajic acid was also found to be indicated at high column temperature as the trimethylsilylated methylester (fig. 13). Noticeably, the Quillajic acid did not appear to be homogeneous but displayed at least two bulges. It is not clear whether this was possibly induced by the isolation and analysis method itself. Indications of the presence of arabinose, glycosylated with two mutually linked fatty acids, were obtained in the analysis of one of the HPLC fractions of QUADRI-2 isolated with mild alkaline hydrolysis. Sugar analysis demonstrated the presence of arabinose (in addition to some xylose and glucose). A direct trimethylsilylation of the fraction followed by GC-MS showed two GC peaks of a glycosylated pentofuranose. The mass spectra of the peaks were in accordance with an arabinose having respectively one and two fatty acids, as proposed by Higuschi et al (in *Phytochemistry* 26, 229-235 (1987)). NMR spectra of the three fragments (Fig. 6, QUADRI-2A, 2B and sugar chain C), prepared as described under example 4, resulted unambiguously in the determination of the sugar structure of fragment QUADRI-2B. Using 2D-NMR the

monosaccharides and their bonds could be identified. The 1D-¹H NMR spectrum of QUADRI-1B was found, as expected, to be identical to QUADRI-2B. It is thus determined that, certainly in respect of the elements xylose, glcA, gal, Quillajic acid, the structure is wholly as proposed by Higuschi et al., and on the basis of FAB-MS also very probably the part bonded to fucose consisting of the double fatty acid glycosylated with arabinose. Much less clear is the situation relating to the remaining sugar part (C).

From the NMR of this fragment (fig. 14), which in the case of QUADRI-2 should consist of apiose, xylose, rhamnose and fucose, there are found to be clearly a number of, at least four, resonances characteristic of a saccharide-methyl group, while only two (fucose, rhamnose) could be present according to the structure of Higuschi. From analysis of the anomeric part of the spectrum there were further found to be 5-6 β -protons (fig. 14), where the structure proposed by Higuschi predicts only 2-3 β -protons (fucose can have an anomeric α -proton or a β -proton after hydrolysis).

On the basis of analogy with the inhomogeneity, mentioned in the international patent application WO 93/05789 of Cambridge Biotech Corp., of their QS(QA) 21, wherein QA-21-V2 was found to contain xylose instead of apiose, this can result in an additional β -proton resonance. This is however not sufficient to explain the spectrum. HPLC separation on a special Dionex column indicated the presence of different peaks and smaller peaks (fig. 15) whereof peaks 2, 3 and 4 were also found to contain sugar.

NMR analysis of the highly diluted solutions led to the conclusion that peak 2 contained a trisaccharide, peak 3 contained a tetrasaccharide in which no apiose was detectable, and peak 4 contained all elements pointing to the presence of the apiose, xylose, rhamnose and fucose present in the predicted structure.

Sugar analysis of these peaks demonstrated that peak 2, while indeed not containing any apiose, does contain 1.0 xylose and 0.9 fucose in comparison to rhamnose and

therefore corresponds with a trisaccharide consisting of xylose, rhamnose and fucose.

Peak 3 showed 1.9 xylose in comparison to rhamnose and therefore corresponds with the residue associated with QA-
5 21-V2 consisting successively of xylose, xylose, rhamnose and fucose. Finally, peak 4 also corresponds in sugar analysis with the structure proposed by Higuchi of successively apiose (0.8), xylose (1.3), rhamnose (1.0) and fucose (0.9). The three peaks correspond in area with a
10 presence of 22% trisaccharide, 17% tetrasaccharide xylose, xylose, rhamnose, and fucose, 61% tetrasaccharide apiose, xylose, rhamnose and fucose.

This shows that an unambiguous substance can still not be isolated even with the current RP-HPLC and that at least
15 three molecules are probably present, one not previously demonstrated and therefore new, namely one wherein the terminal apiose or xylose is wholly absent. This compound is further designated with the term "QUADRI-2tri". That this structure was created as a result of the hydrolysis is
20 not very probable for chemical reasons. It is therefore assumed that this is a new compound.

It is found from the NMR analysis of the hydrolysis fragments that different fragments of the same starting preparation which have a slightly differing elution time on
25 RP-HPLC, namely peak 5 and 6, respectively peak 7 and 8 of fig. 16, display in NMR the same spectra of fragment QUADRI-2B (peaks 7 and 8) respectively of QUADRI-2A (peaks 5 and 6). A separate observation is possibly an explanation for the inhomogeneity. Spectra of QUADRI-1 and QUADRI-2 in
30 D₂O were found to be greatly widened, due to probable micelle formation in water. Addition of deuterated methanol resulted in spectra very strongly similar to previous spectra recorded in d₆-DMSO. There was however a characteristic difference at the position of the aldehyde
35 proton which belongs in the Quillajic acid ring (fig. 17). After dissolving in D₂O the spectrum displayed two aldehyde peaks and a bulge. The relative intensity of the peaks changed as more methanol was added. When this preparation

was subsequently dissolved in DMSO by complete replacement of the solvent, the two peaks with bulge were found to be still present. Direct dissolving of fresh QUADRI-2 in DMSO again resulted in one aldehyde peak (fig. 18). QUADRI-2A also displayed this characteristic, while the spectrum of fragment QUADRI-2B always displayed one aldehyde peak, also in D₂O. A NOE 2D-NMR of the fragment of QUADRI-2A showed in respect of sugar residues no other adjacent group than the glucuronic acid. What was noticeable here however was that in water/methanol both aldehyde resonances displayed NOE in conjunction with two β -resonances of the glucuronic acid. This shows that two conformers are present which, once formed, are reasonably stable. A possible explanation for this phenomenon is that the sugar chain (C) apiose (xylose), xylose, rhamnose, fucose forms a complex with the Quillajic acid part, possibly supported by an intermediary form of a type of acetal bond of the aldehyde group with an OH of the sugar chain, although no support is found for this latter in the 2D-NMR. The observed conformers are also possibly the reason for the inhomogeneity displayed by the GC-MS of Quillajic acid and the fact that in alkaline hydrolysis separation on RP-HPLC often shows two adjacent peaks which are found to be identical in NMR analysis.

This also shows that the position of the RP-HPLC peak in the elution pattern is not unambiguously associated with one substance. The UV/vis-spectrum (fig. 19) shows one clear sharp band at 230 nm and a wide peak at around 280 nm. These bands are also detected in CD (fig. 20). Some inhomogeneity was only found in fluorescence analysis (fig. 21).

The emission spectrum has a maximum at about 350 nm but is quite wide. Excitation spectra recorded at two emission wavelengths (394 and 350 nm) therefore show differences. When the intensity is recorded at 394 nm (in the edge of the emission) the excitation spectrum displayed a clear shift to higher wavelength. Once again this can be explained by the presence of an internal complex. On the basis of this data it is proposed here that QUADRI-2 is

characterized by a constant part formed by QUADRI-2B, to which via the fucose is bonded the double fatty acid residue with the arabinose thereon. This part possibly forms the essential component of the molecule resulting in the adjuvant activity.

The remaining part (C) of the molecule is not homogeneous either in composition or in its spatial structure, but in terms of the internal sugar chain C consists of respectively apiose, xylose and rhamnose, respectively xylose, xylose, rhamnose, fucose, respectively xylose, rhamnose, fucose, together referred to as QUADRI-2. The individual substances are not separated with the RP-HPLC purification method known for this type of substances, whereby internal complex formation can result in at least two peaks in RP-HPLC and whereby this technique is already per se unable to unambiguously define QUADRI-1 and/or QUADRI-2.

QUADRI-1 is possibly also an adjuvant because of the identical nature of the fragments QUADRI-1B and QUADRI-2B and the fatty acid part. In addition to apiose, xylose, rhamnose and fucose, glucose also occurs in this molecule. The substance is however, when isolated, much more toxic and therefore less suitable. Possible changes resulting from the presence of the glucose on the spatial structure could result in this increased toxicity in water compared to QUADRI-2, but this phenomenon is not yet fully understood.

EXAMPLE 3

Toxicity and adjuvant activity of QUADRI-1 and QUADRI-2

Compared to the known commercial Quil A from Superfos "New Quil A" contains a relatively large amount of QUADRI-1 and -2. The toxicity of "New Quil A" was compared by subcutaneously injecting two groups, each of 8 Balb/C mice, with 50 µg per animal dissolved in 0.2 ml phosphate buffer. In the case of Quil A, 88% of the animals died and in the case of "New Quil A" 63%. The animals moreover exhibited much stronger signs of illness than in the case of "New

Quil A". In a hemolysis test (radial diffusion in a gel) QUIL A from Superfos was found to display a 50% higher activity than "New Quil A". This means that, despite the larger quantity of active components QUADRI-1 and -2, "New Quil A" is less toxic than QUIL A. The adjuvant activity of both substances was also compared. Ovalbumin was used as antigen. Both QUIL A and "New Quil A" had roughly the same adjuvant activity in respect of ovalbumin, wherein 10 μ g adjuvant resulted in an approximately 10 times higher titre in an ELISA assay of the antibodies against ovalbumin. It must be noted herein that ovalbumin is a soluble protein which occurs in the cytosol, of which it is known that saponins in that case offer fewer advantages than other adjuvants than in the case of glycosylated proteins which occur in the membrane. In view of the effectiveness of QUADRI-2 as adjuvant of glycoprotein D2 further discussed in this text, where 10 μ g results in a thirty-fold increase in the antibody titre after the first injection and a thousand-fold increase after the "booster", it is self-evident that, in the case of glycosylated proteins and vaccines where the antigen must pass through the membrane, "New Quil A" with its higher concentration of QUADRI-1 and -2 does better as adjuvant than QUIL A. A comparison of the effect of diverse adjuvants is developed further on in this example.

50, 100, 200, 400 or 800 μ g QUADRI-1 respectively QUADRI-2 were administered subcutaneously to groups of 6 mice in each case. Even the maximum dose used of 800 μ g QUADRI-2 per mouse of 20 g was found not to have lethal consequences for any of the 6 test animals. This means that the LD₅₀ of QUADRI-2 is greater than 40 mg/kg test animal. In the case of QUADRI-1 an LD₅₀ of approximately 7.5 mg/kg was found. QUIL A® from Superfos was used as reference material. The LD₅₀ of this preparation amounted to approximately 25 mg/kg. It must however be noted herein that the amount of effective substance in the case of QUADRI-1 and QUADRI-2 is much higher than in QUIL A®. QUIL A® contains only 5% effective saponins. A dose of 1 mg/kg QUADRI-1

therefore corresponds to 20 mg/kg QUIL A®. This shows that the toxicity of the pure QUADRI-1 and QUADRI-2 compounds is much lower than the toxicity of the commercially available QUIL A preparation.

- 5 Hemolysis of QUADRI-2 in a classical red blood-cell test indicated 5 µg/ml. A more sensitive test, wherein the disruption of the membrane is followed by the release of a fluorescent marker, showed that the cell lysis was complete at 5 µg QUADRI-2.
- 10 Adjuvant activity was compared for QUADRI-1 and 2 by administering 100 µg ovalbumin in the presence of differing amounts of adjuvant to different groups of guinea-pigs (fig. 22). As can be seen in fig. 22, 125 µg QUADRI-2 gives approximately a hundred-fold increase in the titre of
- 15 antibodies against ovalbumin. 25 µg has roughly the same effect. If QUADRI-2 is used as component of the ISCOM matrix there is then found to be a maximum (again about a hundred-fold) with the use of 25 µg QUADRI-2. The avidity of the generated antibodies against ovalbumin is also found
- 20 to differ when QUADRI-2 is added not separately but in the ISCOM matrix. By means of a displacement reaction with ammonium isothiocyanate the generated antisera of the ISCOM matrix are found to result in clearly more strongly binding antibodies.
- 25 In order to compare the effect of diverse adjuvants experiments were done with groups of in each case 5 mice, again with ovalbumin as antigen. QUADRI-1 and -2 (5 and 25 µg, freely and as ISCOM) were compared with QUIL A, Al(OH)₃ and incomplete Freund's adjuvant. Not only was the titre
- 30 measured but also the type of antibodies that were generated. The results are collated in Table 3. On the basis of the total concentration of Ig-antibodies, Al(OH)₃ and IFA appear to be still better than saponin. However, only QUADRI-1 and -2 in the ISCOM form generate the
- 35 important IgG2a-antibodies and not the hypersensitive IgE-antibodies as in the case of Al(OH)₃ and IFA.

Table 3

Group	adjuvant	Total Ig	IgG1	IgG2a	IgG2b	IgE
1	none	1.5	1.5	-	-	-
2	Al(OH) ₃	3.5	3.7	-	-	1.2
3	FCA/FIA	4.2	4.0	1.1	1.9	1.5
4	5µg Superfos Quil A	1.5	1.3	-	-	-
5	25µg Superfos Quil A	2.4	1.8	-	-	-
6	1µg q1F	1.2	-	-	-	-
7	5µg q1F	1.8	1.7	-	-	-
8	25µg q1F	*	*	*	*	*
9	1µg q1M	2.0	2.0	-	-	-
10	5µg q1M	2.6	2.2	1.4	-	-
11	25µg q1M	2.7	2.4	1.5	-	-
12	1µg q2F	-	-	-	-	-
13	5µg q2F	1.8	1.7	-	-	-
14	25µg q2F	2.2	2.0	-	-	-
15	1µg q2M	1.9	1.9	-	-	-
16	5µg q2M	2.7	2.8	1.2	-	-
17	25µg q2M	2.6	2.5	1.3	1.5	-

20 q1F: QUADRI-1, free form

q1M: QUADRI-2, matrix form

q2F: QUADRI-2, free form

q2M: QUADRI-2, matrix form

-: titer lower than 1.0

25 *: the mice could not stand 25µg pure QUADRI-1
and died.

If a glycosylated antigen is used as glycoprotein D2 (gD) instead of ovalbumin, the effectiveness of QUADRI-2 is then found to be very great compared to $\text{Al}(\text{OH})_3$. In this experiment 10 Balb/C mice were injected with 1 μg in combination with 1-10 μg QUADRI-2 (in free form). After 14 days the mice were injected again with the same mixture ("booster"). Titres were measured after 14 days and 21 days. After the first injection a quantity of 5 μg QUADRI-2 was found to be necessary to obtain an adjuvant activity of about a factor 5. With 10 μg this could be doubled at minimum (the experiments exhibited a wide divergence, but very significant differences). $\text{Al}(\text{OH})_3$ displayed no action whatever at this antigen concentration of 1 μg . After the "booster" the results were still more dramatic; 5 μg QUADRI-2 was found to have a hundred and fifty-fold effect and 10 μg even gave a thousand-fold increase in the titre. $\text{Al}(\text{OH})_3$ achieved hardly a ten-fold increase after the booster and was only just higher than a blank sample.

The results indicate that QUADRI-2 is a very good adjuvant with a low toxicity and is possibly still more effective as component of the ISCOM matrix. Saponins are more effective in the case of glycosylated proteins. "New Quil A" as such will in many cases be a better adjuvant than the classical QUIL A due to a lower toxicity and the higher concentration of QUADRI-2.

EXAMPLE 4

Hydrolysis of QUADRI-1 and QUADRI-2

In order to be able to prove the structure of QUADRI-1 and QUADRI-2 hydrolysis tests are done with the pure substances QUADRI-1 and QUADRI-2. QUADRI-1 is dissolved in an alkaline NaOH solution with a pH of 12 at room temperature (so-called mild hydrolysis). After 30 minutes the hydrolysis is stopped and the reaction mixture analyzed on RP-HPLC (VYDAC, C_4 -column, eluted with a 0.15% trifluoroacetic acid solution in water, combined with an acetonitrile gradient of 30%-45%). It is found here that QUADRI-1 is converted for the most part into a new compound which is character-

ized by a retention time of about 8 minutes (see figure 7). This compound is the hydrolysis product QUADRI-1A. 30% acetonitrile is then added to an alkaline solution (pH 12) of QUADRI-1, which solution is heated for 16 hours at 100°C
5 (so-called "strong hydrolysis"). The elution pattern then shows, in addition to the peak of the void volume, two other peaks (see figure 9), the first of which, with a retention time of about 8.7 minutes, corresponds with the peak occurring after the mild hydrolysis (compare figure
10 7), and the second of which corresponds with QUADRI-1B.

Similar experiments are performed with QUADRI-2. The product resulting from QUADRI-2 after a mild hydrolysis is characterized by a retention time of about 9.6 minutes (fig. 8), while after strong hydrolysis the product QUADRI-
15 2B has the same retention time as QUADRI-1B (results not shown). In order to compensate for the differences in the elution times the strongly alkaline hydrolysates of QUADRI-1 and QUADRI-2 are mixed and placed on a column. Figure 10 shows that only three peaks can be seen. These peaks are
20 QUADRI-1A (8.645 minutes), QUADRI-2A (10.363 minutes) and QUADRI-1B and -2B together (13.109 minutes). The results found are in accordance with the fact that QUADRI-1 has one glucose more on a part that is not yet cleaved after mild hydrolysis but is cleaved after strong hydrolysis. The
25 products remaining after strong hydrolysis are therefore identical, while the products differ mutually after mild hydrolysis.

A sugar analysis was performed on the hydrolysis products of QUADRI-1. The results hereof can be seen in
30 table 2 (above).

The fact that these compounds, which can also be found in QUIL A which is extracted from old bark, can be generated from two compounds QUADRI-1 and QUADRI-2 was previously unknown.

35 Old bark often comprises a duplication of peaks leading to the complex pattern as represented in fig. 3 for old bark. In order to study whether this duplication could be explained from a permanent form of acetal formation as

proposed in example 2 as an intermediate, non permanent structure, a preparation of old barked was purified into essentially the QUADRI-1 and QUADRI-2 peak, respectively. This QUADRI-2 shows on RP-HPLC two peaks of 25.7 and 26.4 minutes, respectively. After strong alkaline hydrolysis leading to fragment QUADRI-2B there appeared to be still two peaks as shown in fig. 23. This means there exists an inherent duality which can not be explained by means of internal crosslinking, but that there exists probably a difference in composition between the two components of the "double peak". From this it follows that normal QUADRI-2 is less suited to isolate relatively pure QUADRI-2 therefrom because of the inherent presence of two compounds, which are very similar, and which duality is not present in "New Quil A".

EXAMPLE 5

ISCOM formation

A solution of one or more of the compounds according to the present invention is prepared by dissolving a number of mg of the compound in a corresponding number of ml of water (this is solution A).

Phosphatidylcholin is dissolved in water with 20% of the detergent Mega-10® (Sigma) to a concentration of 10 mg/ml. 10 mg cholesterol per ml is dissolved therein by stirring at 50°C. This is solution B.

An antigen solution is obtained by dissolving a determined number of mg of antigen in a corresponding number of ml of water. This is solution C.

To form an ISCOM matrix 20 μ l B is added per ml A. ISCOMS are prepared from 1 ml A, 1 ml C and 20 μ l B. The mixtures are shaken on a tumbler for 3 to 4 hours. If necessary the Mega-10® is removed by dialyzing against physiological salt solution (0.85% NaCl, 0.01 M phosphate buffer, pH 6.5-7.2).

Figure 24 is an electron microscopic photo illustrating the ISCOM-forming properties of QUADRI-2. Figure 25 shows the ISCOM matrices formed with one of the two hydro-

lysis products obtained after mild hydrolysis of QUADRI-1 (the product which showed a value of 2.405 in the sedimentation experiments of figure 28). The hydrolysis products are found to form smaller ISCOM matrices than the starting products.

Using the "Zetamaster" of Malvern Instruments Ltd., England, the so-called Zeta potential is measured. The Zeta potential shows the stability of a particle in a solution. With the ISCOM matrices with compounds according to the invention in a physiological salt solution (0.85% NaCl, 0.01 M phosphate buffer, pH 7.2) a value of -22 mV was obtained. This means that a very stable micelle (ISCOM) is obtained.

Ultracentrifuge experiments are performed wherein the sedimentation speeds of the diverse ISCOM matrices are detected in generally known manner in a sucrose gradient (figures 26 (QUADRI-2) and 27 (QUADRI-1)). QUADRI-1 and QUADRI-2 form homogeneous ISCOM matrices. The products which are subjected to alkaline hydrolysis for only a half-hour are not homogenous (see figures 7 and 9) and therefore display two peaks in their sedimentation speeds (figures 28 and 29). The absolute value of the diverse sedimentation speeds indicates that ISCOMS of very different size can be made, which is another means of being able to influence the presentation of antigens in controlled manner. This also offers the possibility of selectively entering into interaction with various types of cells or cell compartments. Also significant here is that the ISCOM matrices prepared with hydrolyzed saponins also have the same high stability, as characterized by their Zeta potential of -22 mV.

Because very pure saponins and hydrolyzed and/or modified derivatives thereof are now available, it becomes possible to control the immune response. Depending on the choice of the compound, a wide spectrum of responses, for instance varying between a rapid but possibly less wide and persistent protection to a slower but wider and more pro-

longed protection can be made possible. The formation of antibodies, respectively cytotoxic T-lymphocytes can also be influenced. The individual compounds can likewise be applied in combination with ISCOMS. In addition the ISCOMS
5 can be used on their own.

In summary, it can be stated that "New Quil A" itself and the compounds according to the invention, whether or not they are hydrolyzed and/or modified, can be used independently as adjuvant or be involved in the forming of
10 the ISCOM matrix or ISCOMS. In addition the compounds are suitable for use in hemolysis and they can be used for the targeted release of medicines. In all cases the substances have the advantage that they can be identified as pure components and as a limited group of 3 almost homologous
15 compounds having strongly related properties with respect to adjuvant activity, and in the case of QUADRI-2 show together a low toxicity, because of which QUADRI-2 in particular and/or its components can therefore probably be registered as adjuvant acceptable for use in for instance
20 human vaccines.

The better quality of "New Quil A" and the higher degree of purity of the composition of "New Quil A" and the group of components isolated therefrom will further lead to a low toxicity and fewer side-effects (such as allergy,
25 irritation and the like). This is attributable among other things to the fact that due to the greater purity a lower concentration can be used, but also because contaminants in the existing preparations which can generate such reactions are not or to a lesser extent present in "New Quil A"
30 according to the present invention.

CLAIMS

1. Plant cell extract with adjuvant activity obtainable by extracting fresh plant material consisting of substantially living cells, originating from saponin producing plants.
- 5 2. Plant cell extract as claimed in claim 1, characterized in that the plant material originates from plants of the genera Quillaja, Saponaria or Gypsophilia.
3. Plant cell extract as claimed in claim 1 or 2, characterized in that the plant material originates from
- 10 Quillaja saponaria Molina, or Gypsophilia paniculata, Gypsophilia pacifica, Gypsophilia arrostii, Gypsophilia struthium or Saponaria officinalis.
4. Plant cell extract as claimed in claim 1, 2 or 3, characterized in that the plant material consists of
- 15 cambium cells or meristem cells.
5. Plant cell extract as claimed in claim 1, 2 or 3, characterized in that the plant material is formed by cells from a suspension cell culture.
6. Plant cell extract as claimed in claim 1, 2 or 3,
- 20 characterized in that the plant material is formed by cells from a tissue culture.
7. Plant cell extract as claimed in claim 1, 2 or 3, characterized in that the plant cell material originates from a root culture.
- 25 8. Compounds having adjuvant activity obtainable by separating into its components a plant cell extract according to any one of the claims 1-7.
9. Compounds as claimed in claim 8, characterized in that the separation in its components is effected by means
- 30 of a reverse phase HPLC on a Vydac C4 column with a gradient of 30-45% acetonitrile and 0.15% TFA.
10. QUADRI-1.
11. QUADRI-2.
12. QUADR-2tri.

13. Compounds as claimed in any of the claims 8-12, **characterized in that** they are at least partially hydrolyzed derivatives of the starting product.

14. QUADRI-1A.

5 15. QUADRI-1B.

16. QUADRI-2A.

17. QUADRI-2B.

18. Compounds as claimed in any of the claims 8-17, **characterized in that** they are modified derivatives of the
10 starting product.

19. Method for preparing substantially pure compounds with adjuvant activity as claimed in any of the claims 8-12, comprising subjecting to separation into fractions of a plant cell extract as claimed in any one of the claims 1-7
15 in order to obtain at least one purified component.

20. Method for preparing substantially pure saponin-like compounds with adjuvant activity as claimed in any of the claims 13-17, comprising subjecting to separation into fractions of a plant cell extract as claimed in any one of the claims 1-7 in order to obtain at least one purified
20 component and subsequently at least partially hydrolyzing the purified component(s).

21. Method for preparing substantially pure saponin-like compounds with adjuvant activity as claimed in claim
25 18, comprising subjecting to separation into fractions of a plant cell extract as claimed in any one of the claims 1-7 in order to obtain at least one purified component, optionally hydrolyzing the purified component(s) and subsequently at least partially modifying thereof.

30 22. Method as claimed in claim 21, **characterized in that** the modification consists of linking an antigen to the compound.

23. Method as claimed in any of the claims 19-22, **characterized in that** the separation into fractions is
35 performed by means of preparative HPLC.

24. ISCOM matrix comprising at least one lipid, at least one detergent and at least one plant cell extract as claimed in any of the claims 1-7 and/or at least one

compound as claimed in any one of the claims 8-18, or combinations thereof.

25. ISCOMS comprising the ISCOM matrix as claimed in claim 24 in combination with at least one antigen.

5 26. Adjuvant comprising a plant cell extract as claimed in any one of the claims 1-7 and/or at least one of the compounds as claimed in any of the claims 8-18 and/or an ISCOM matrix as claimed in claim 24 and/or one or more ISCOMS as claimed in claim 25.

10 27. Vaccine comprising an adjuvant as claimed in claim 26 and at least one vaccinating substance.

28. Use of a plant cell extract as claimed in any one of the claims 1-7 in an ISCOM matrix.

15 29. Use of a plant cell extract as claimed in any one of the claims 1-7 in ISCOMS.

30. Use of a plant cell extract as claimed in any one of the claims 1-7 in an adjuvant.

31. Use of a plant cell extract as claimed in any one of the claims 1-7 in a vaccine.

20 32. Use of compounds as claimed in any of the claims 8-18 in an ISCOM matrix.

33. Use of compounds as claimed in any of the claims 8-18 in ISCOMS.

25 34. Use of compounds as claimed in any of the claims 8-18 as an adjuvant.

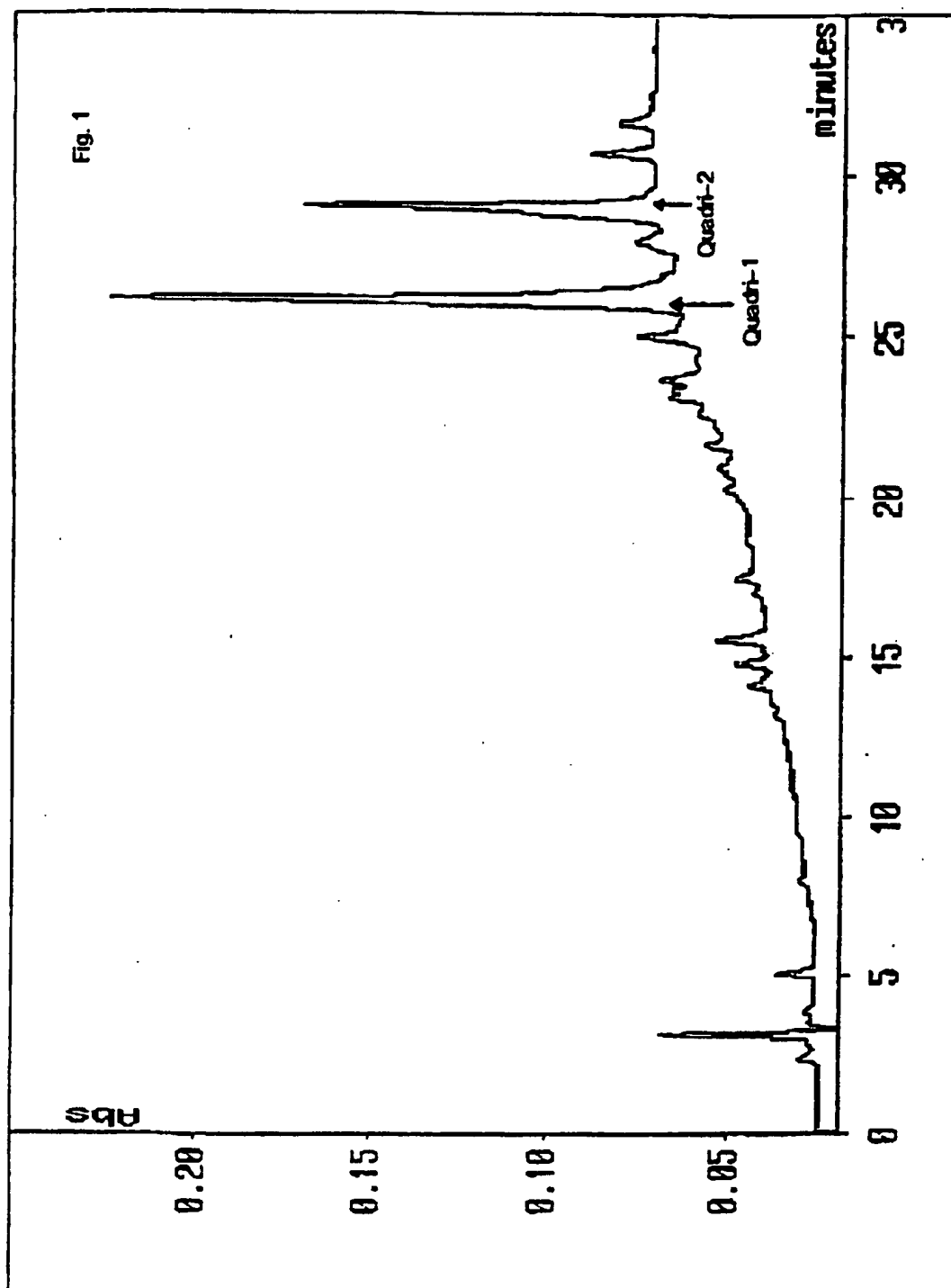
35. Use of compounds as claimed in any of the claims 8-18 in a vaccine.

36. Method for controlling the degree of enhancement of and the type of the immune response by choosing, subject to the desired response, a plant cell extract as claimed in any one of the claims 1-7 and/or a compound as claimed in any of the claims 8-18, whether or not in combination with one or more ISCOM matrices as claimed in claim 24 and/or one or more ISCOMS as claimed in claim 25.

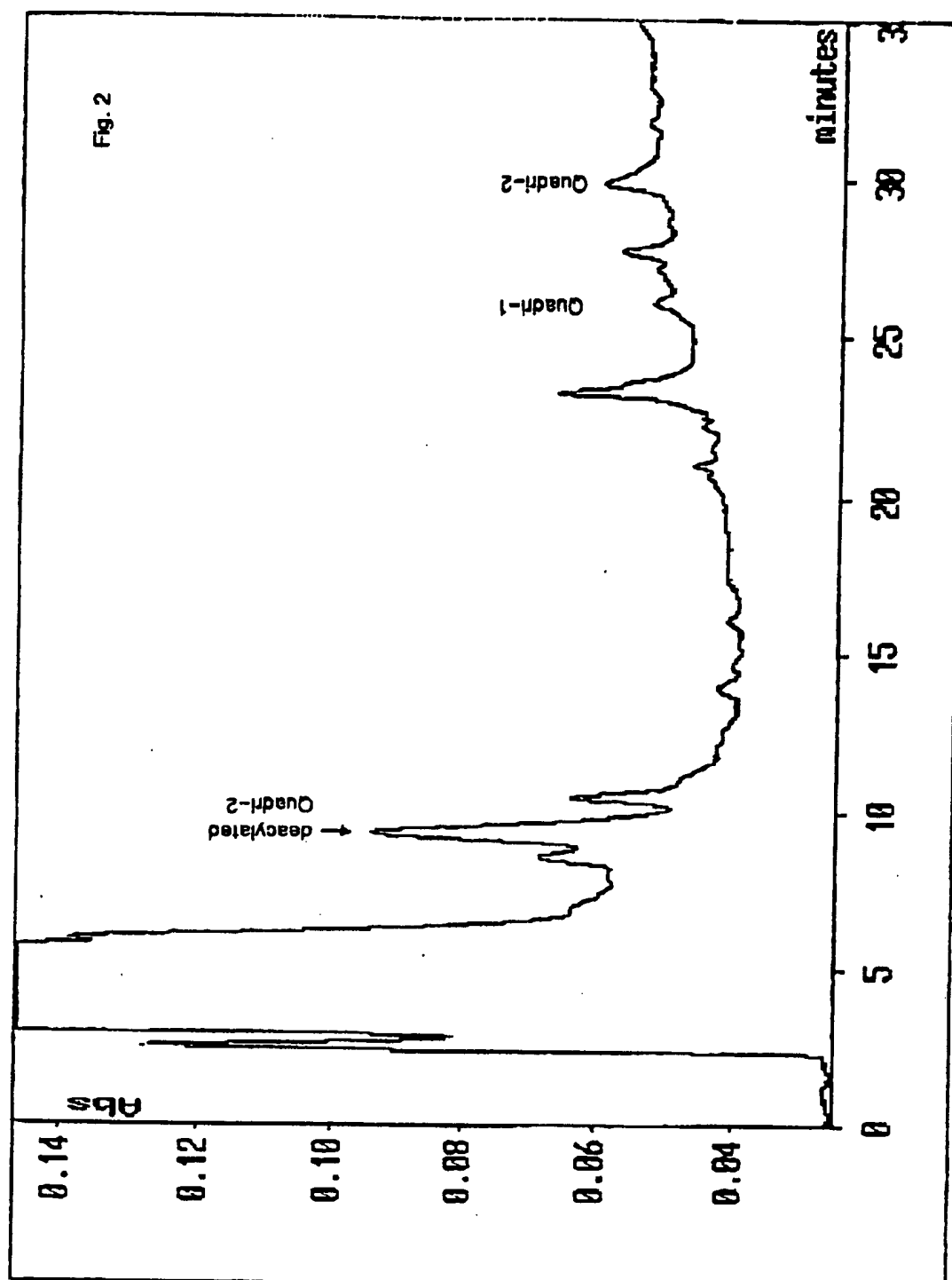
37. Method for hemolysis of for instance red blood cells by adding to the cells a plant cell extract as claimed in any one of the claims 1-7 and/or at least one compound as claimed in any of the claims 8-18.

38. Method for targeted release of a pharmaceutical agent to a particular location in the body by administering to a patient a pharmaceutical agent included in an ISCOM matrix as claimed in claim 24, wherein in and/or on the
- 5 ISCOM matrix is also optionally included a molecule targeted to the particular location in the body.

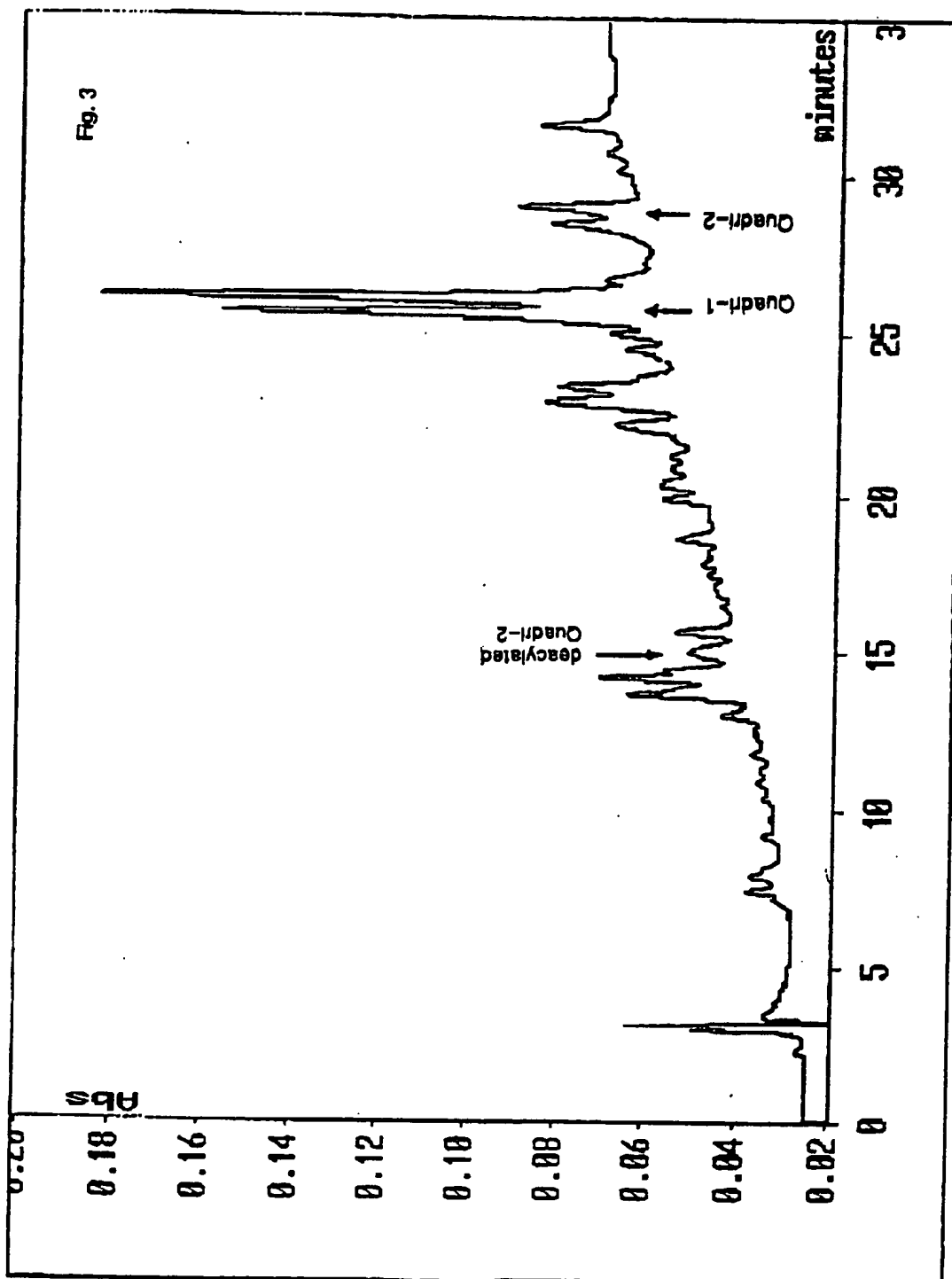
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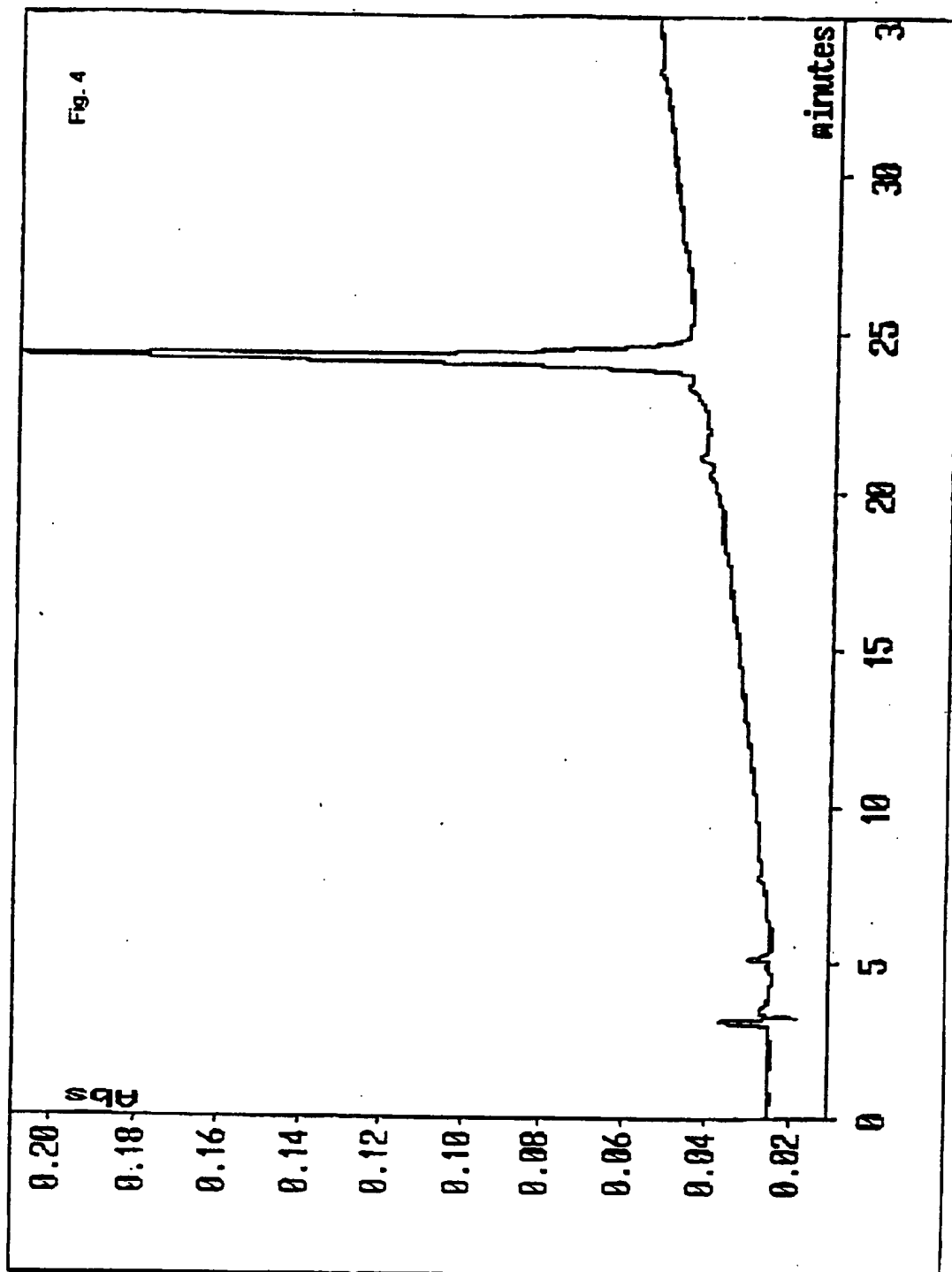
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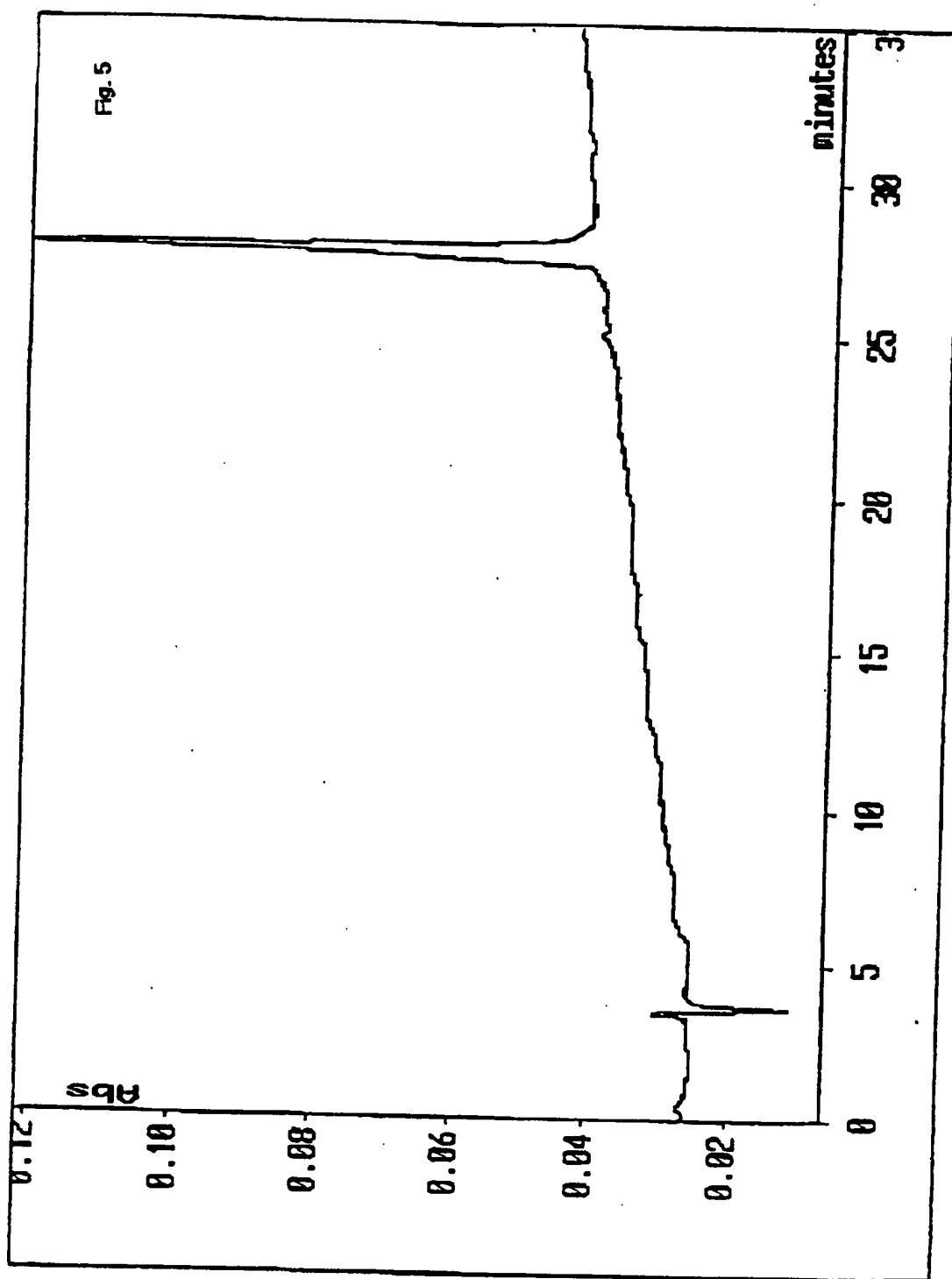
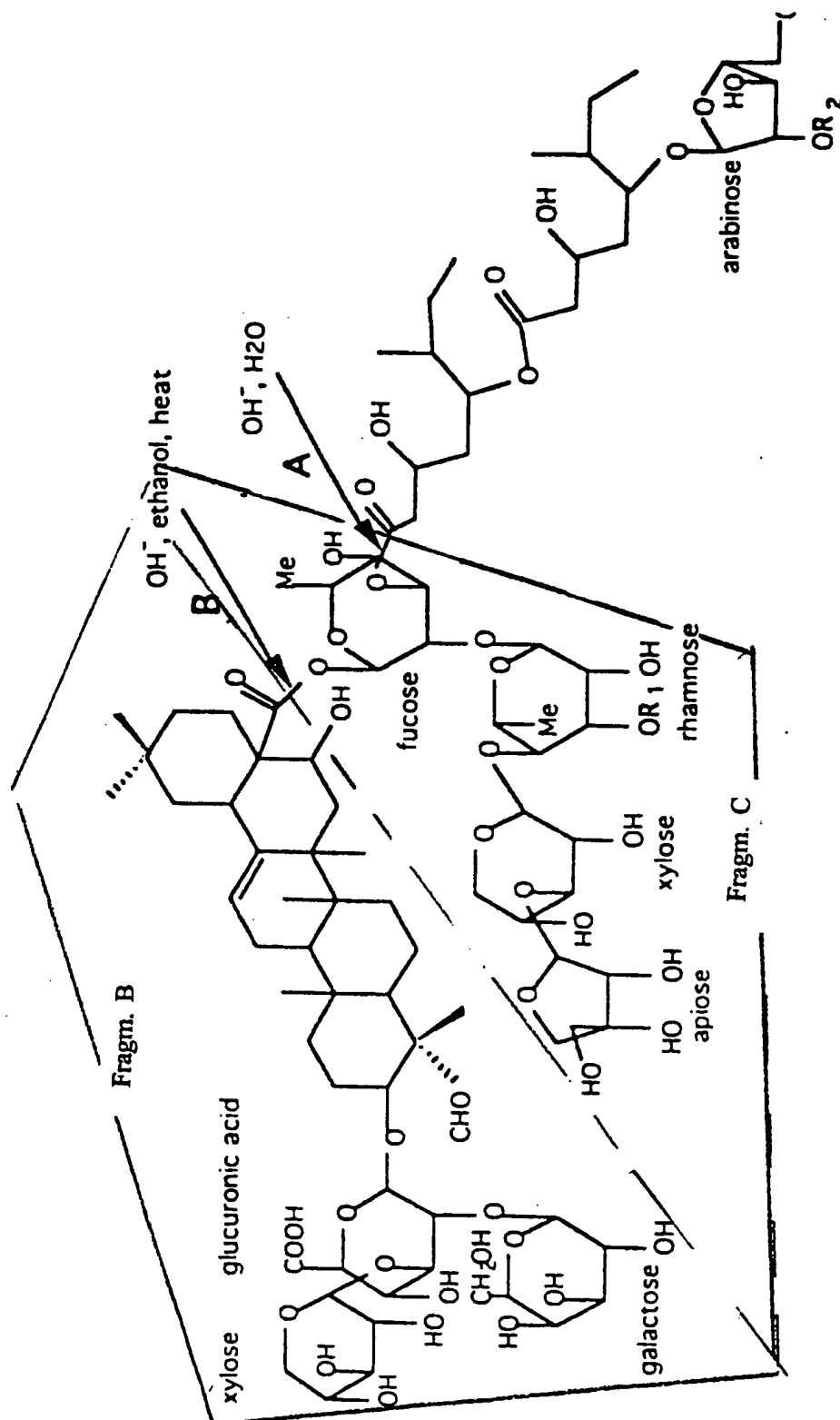
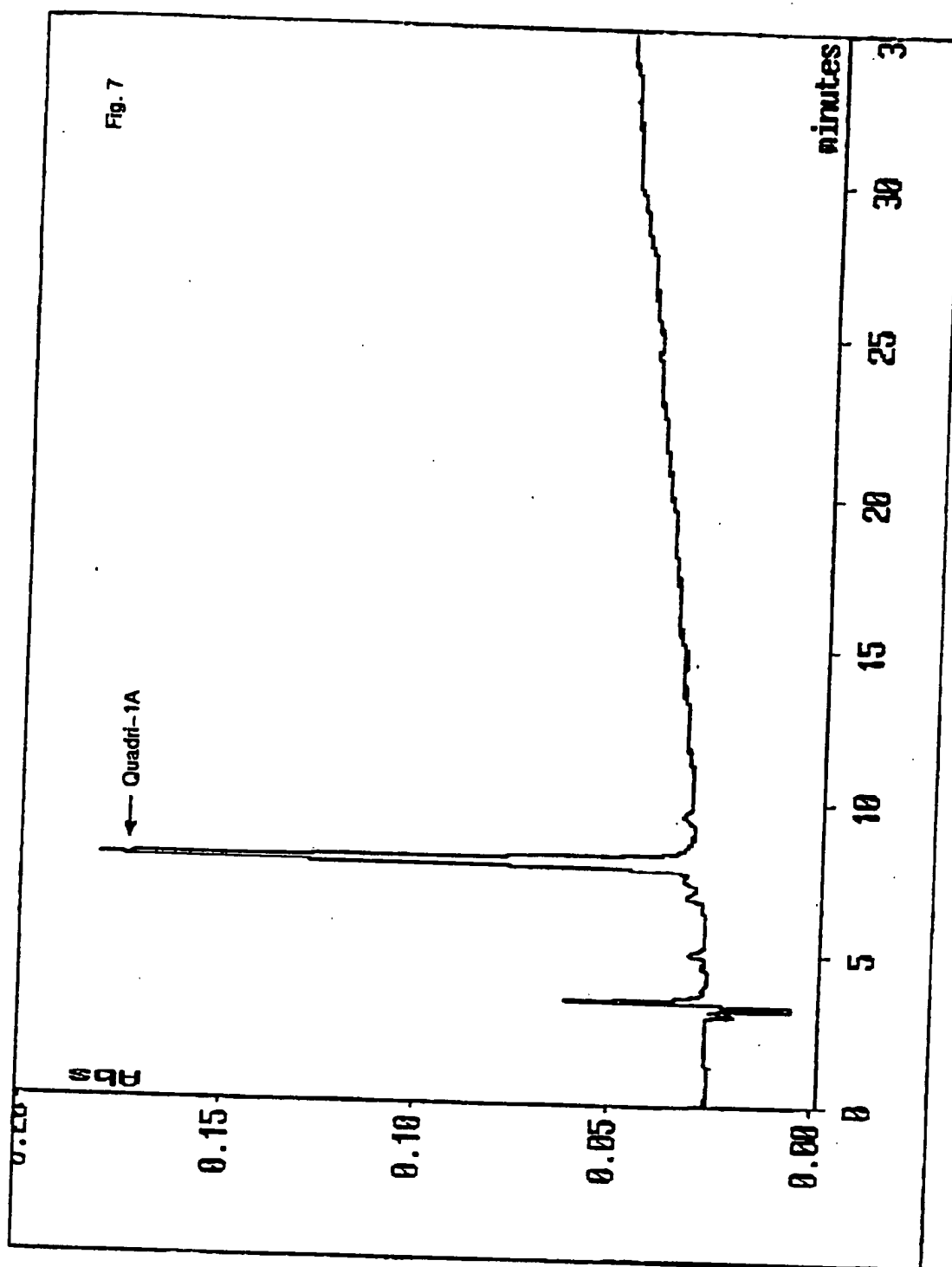


Fig. 6

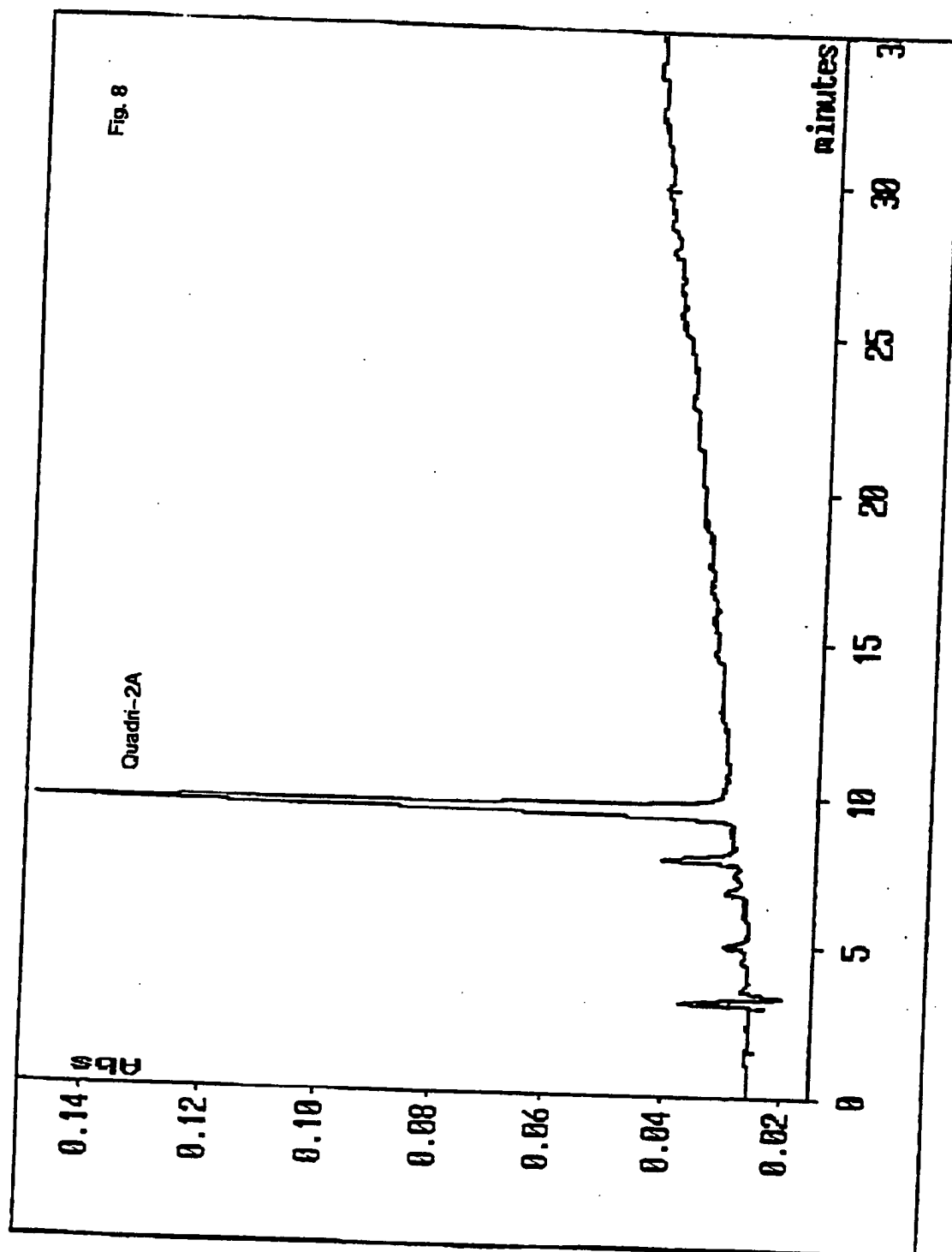


Fragm. A = B + C

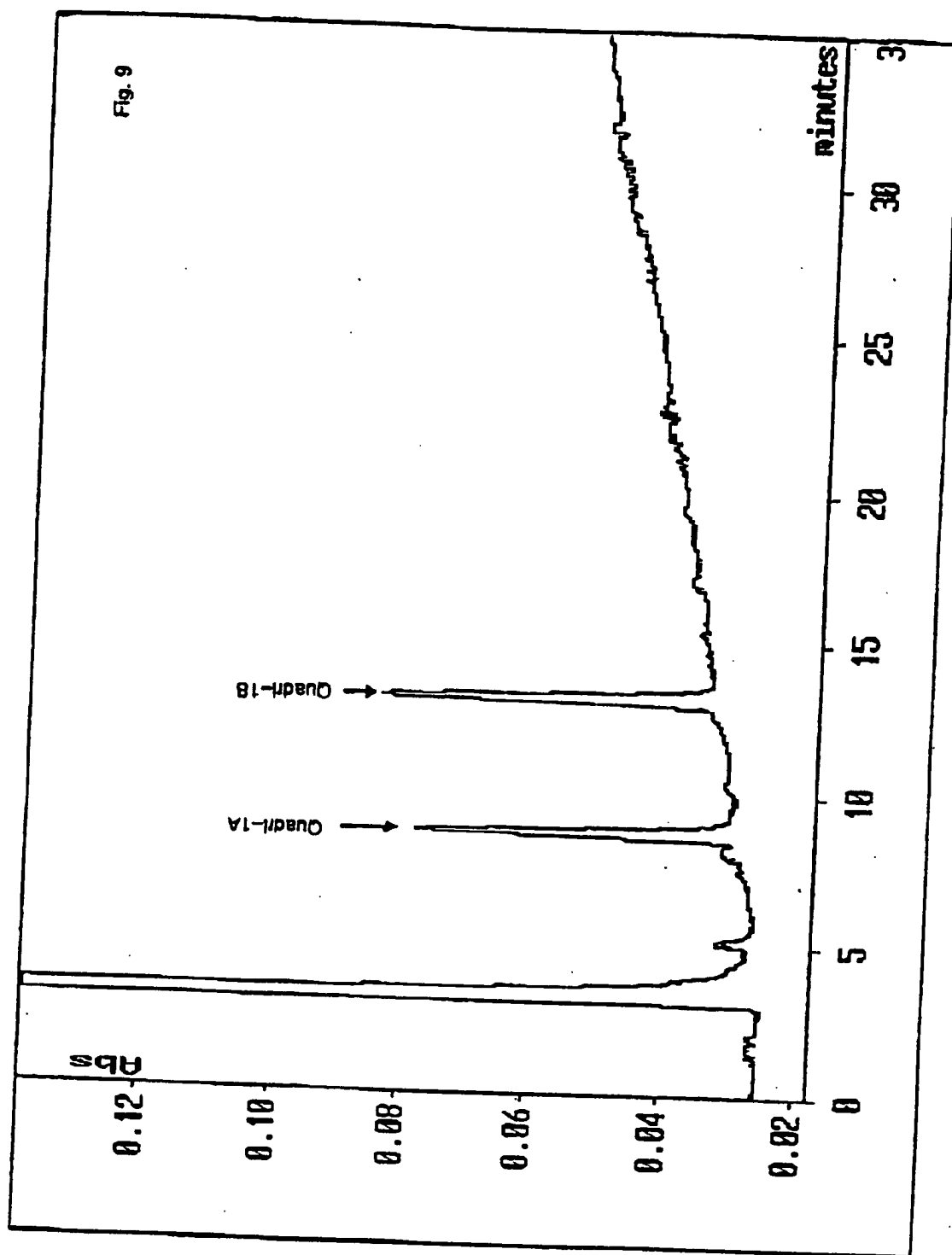
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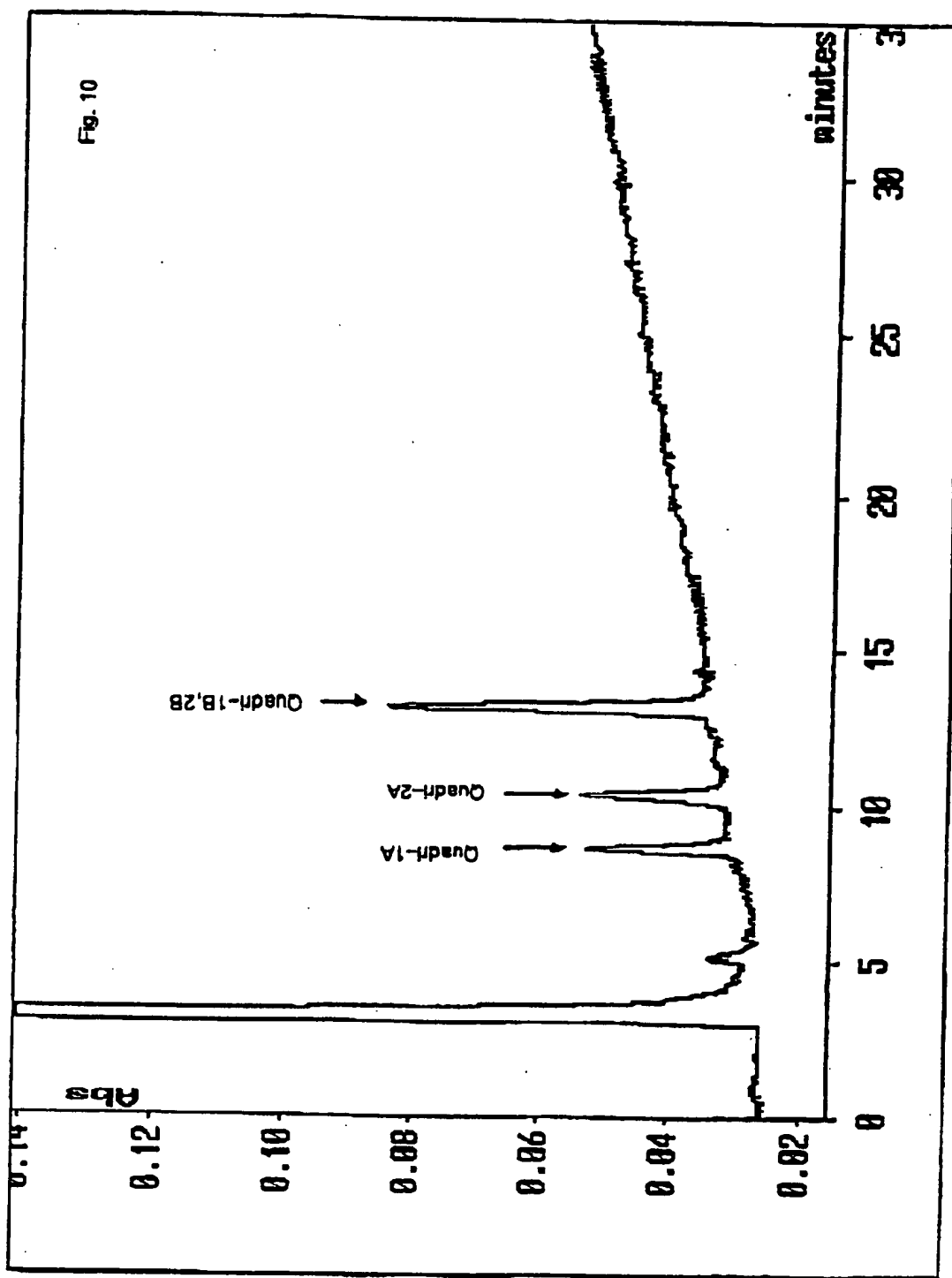
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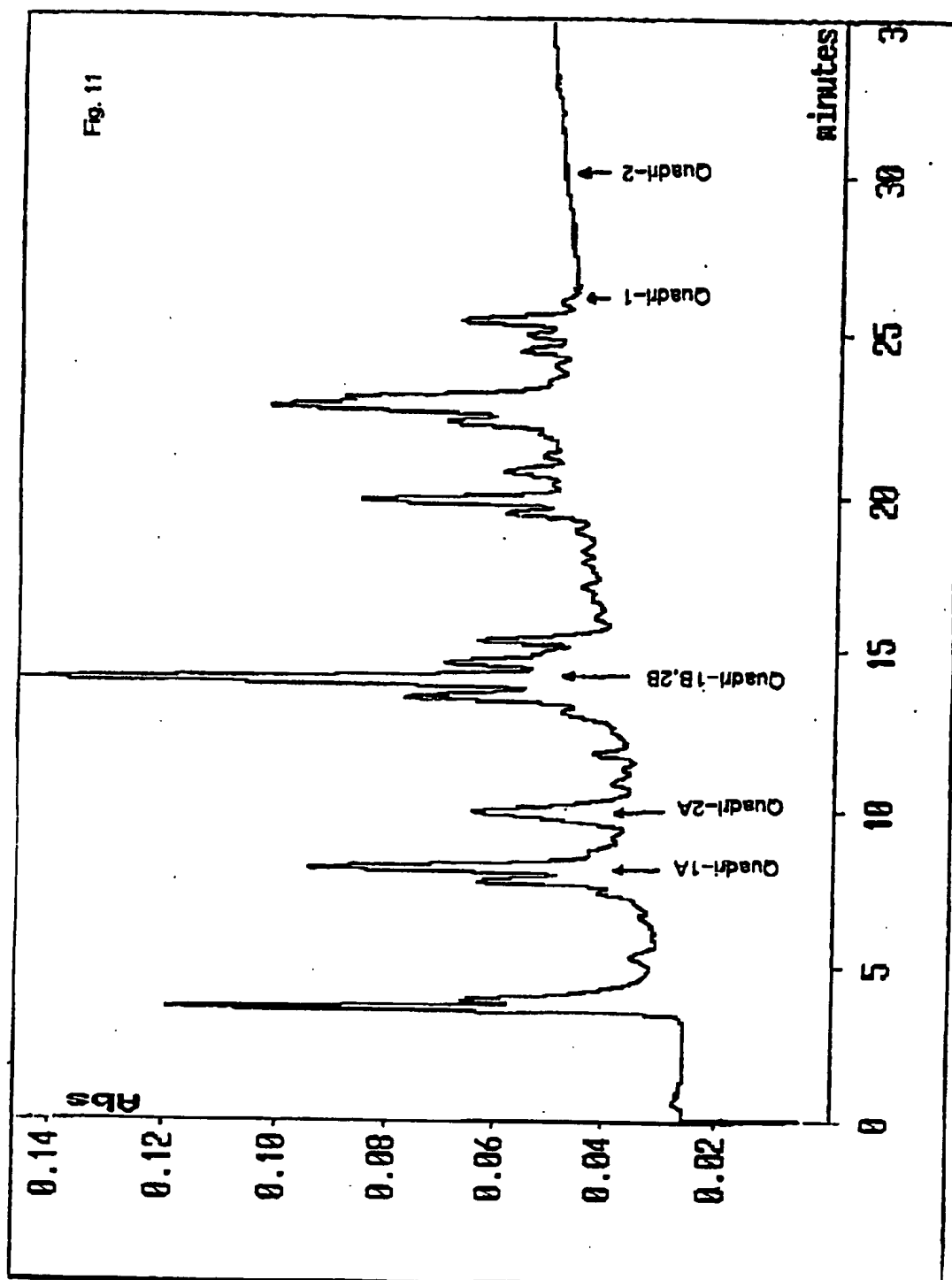


Fig. 12

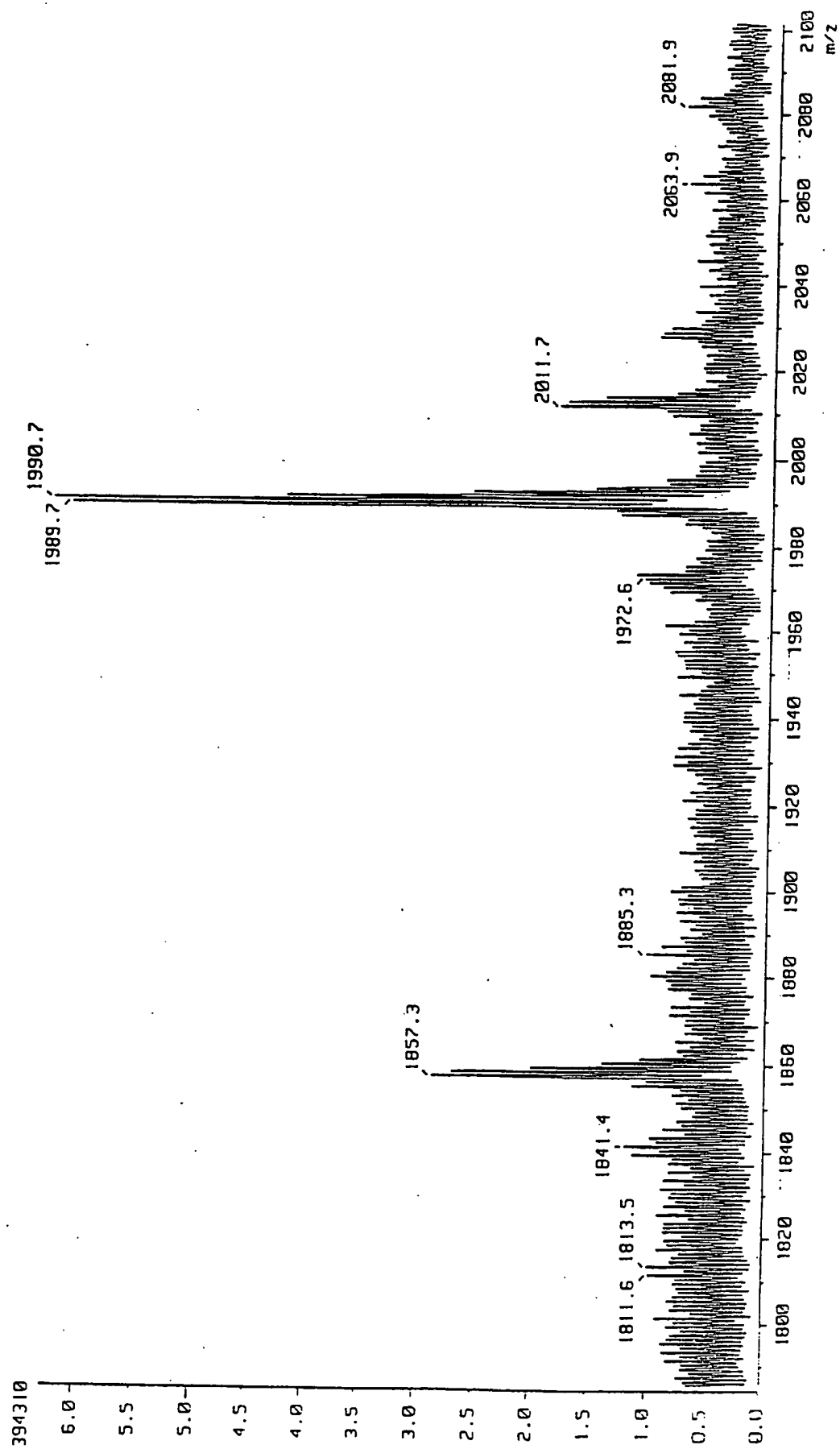
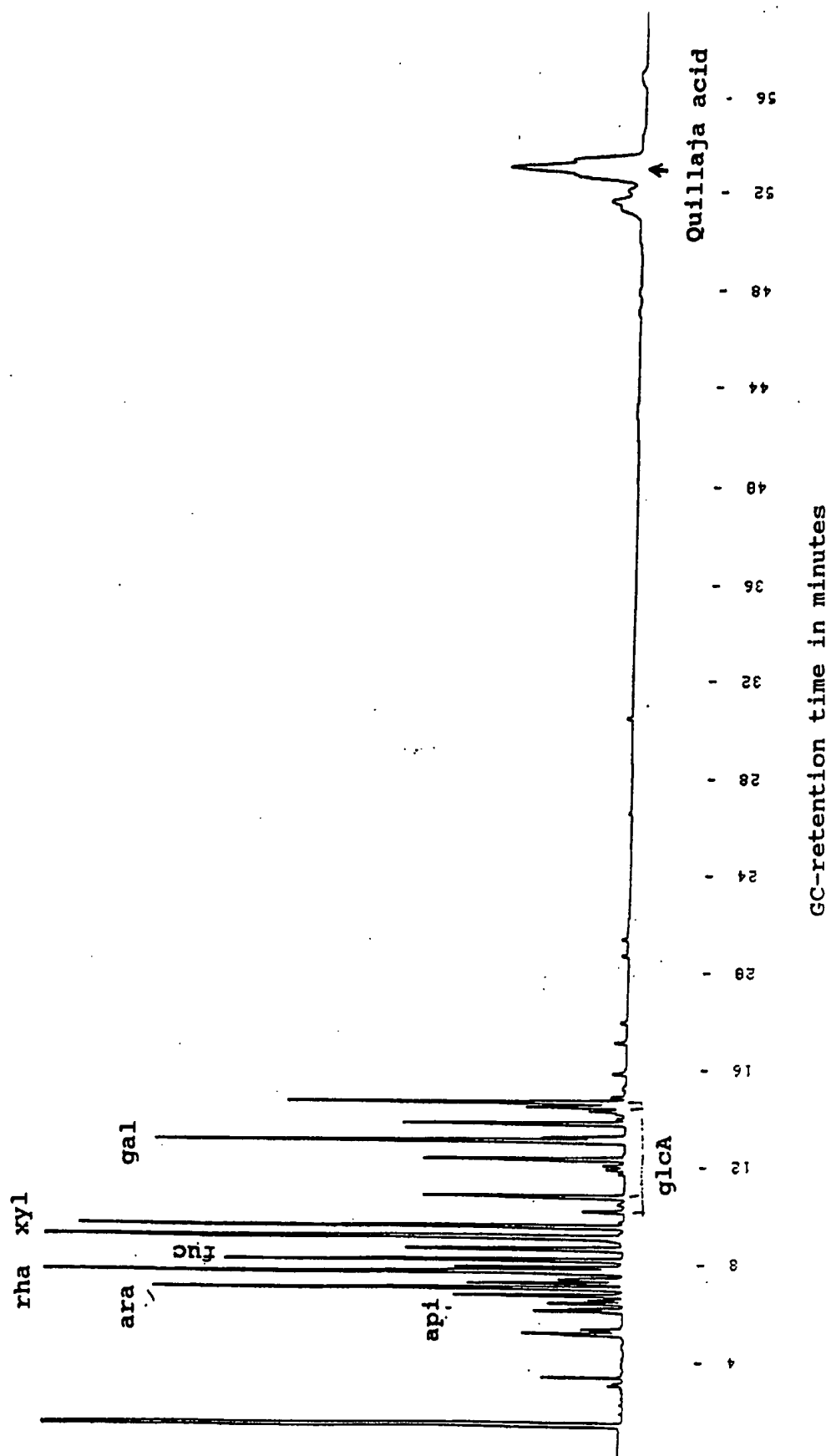
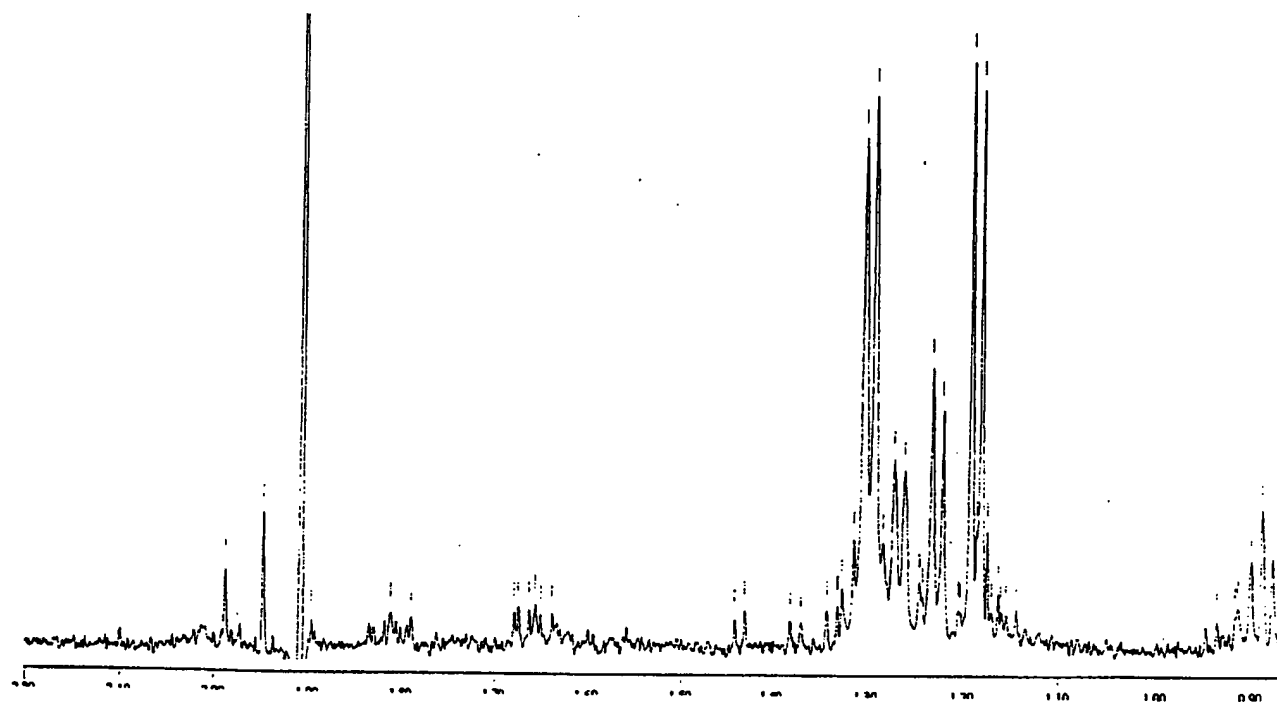
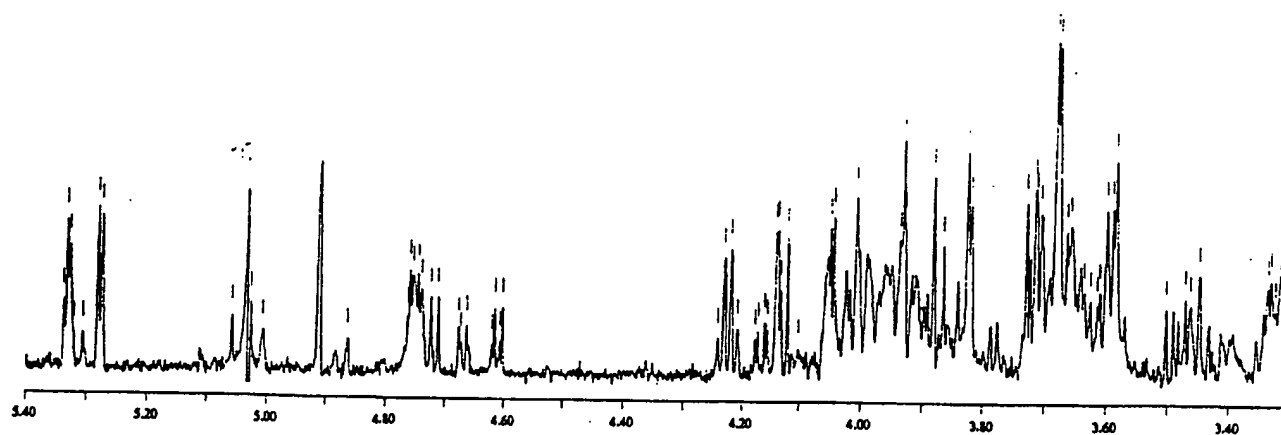
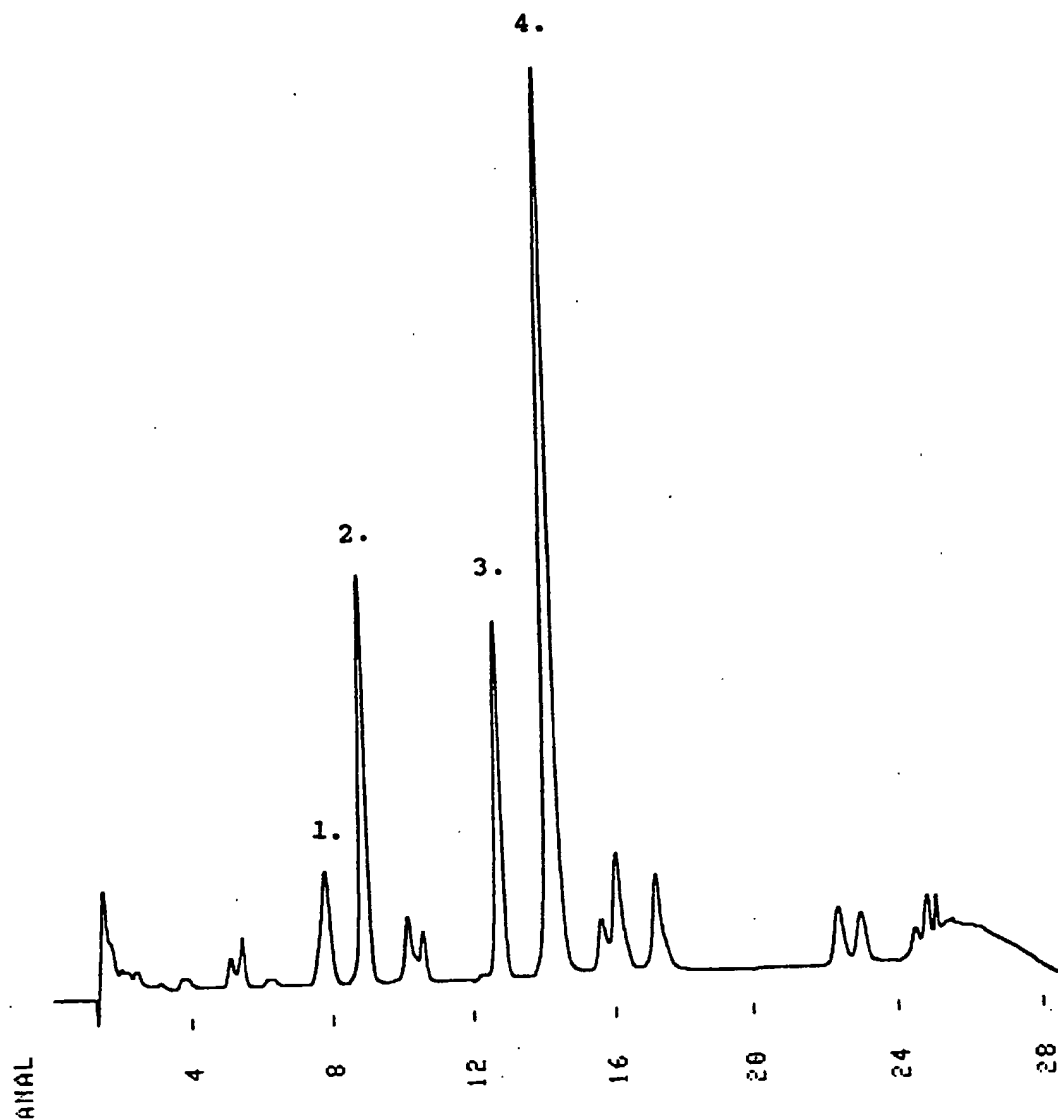


Fig. 13



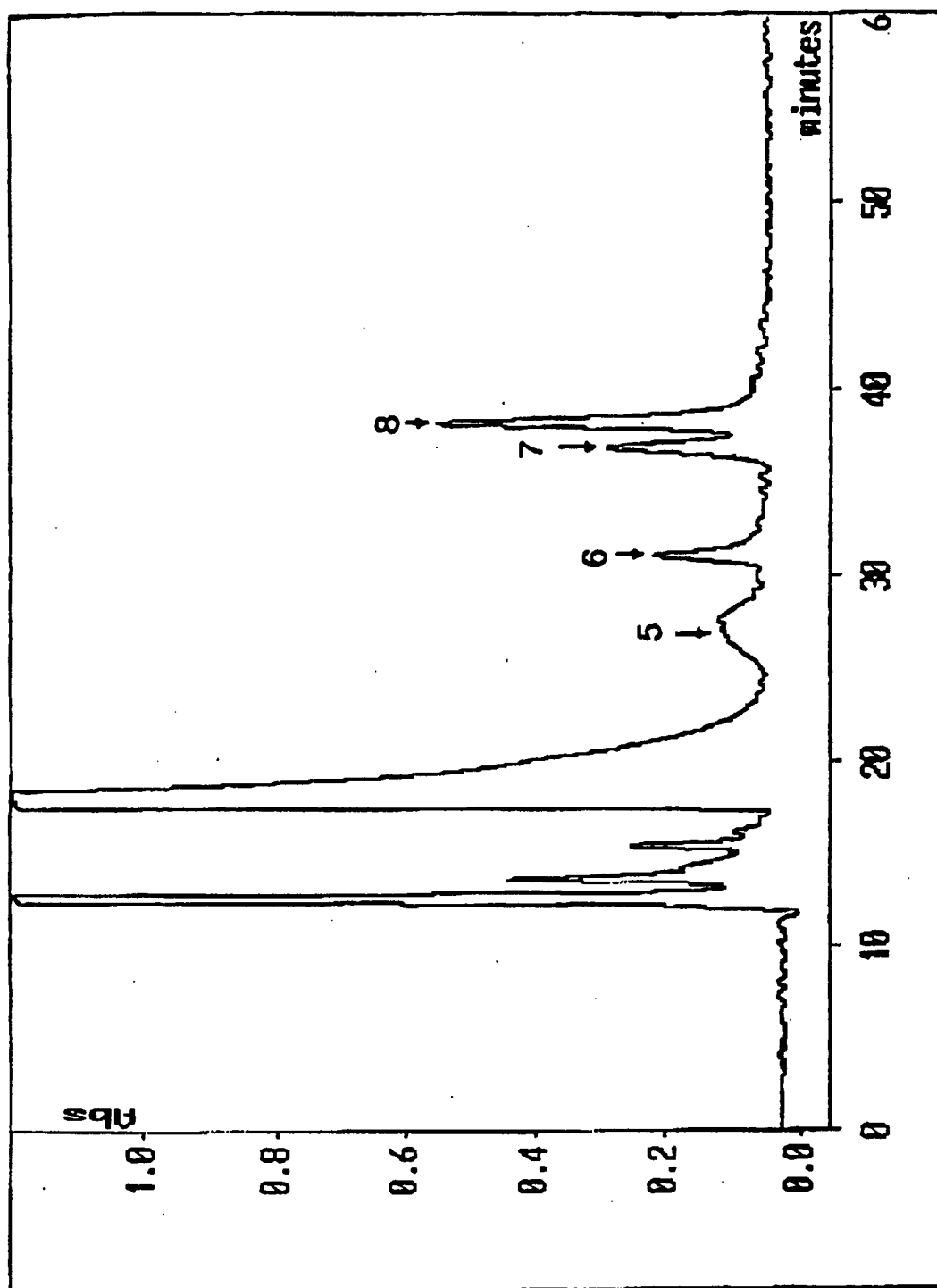


Fragment B



GC-retention time in minutes

fig. 16



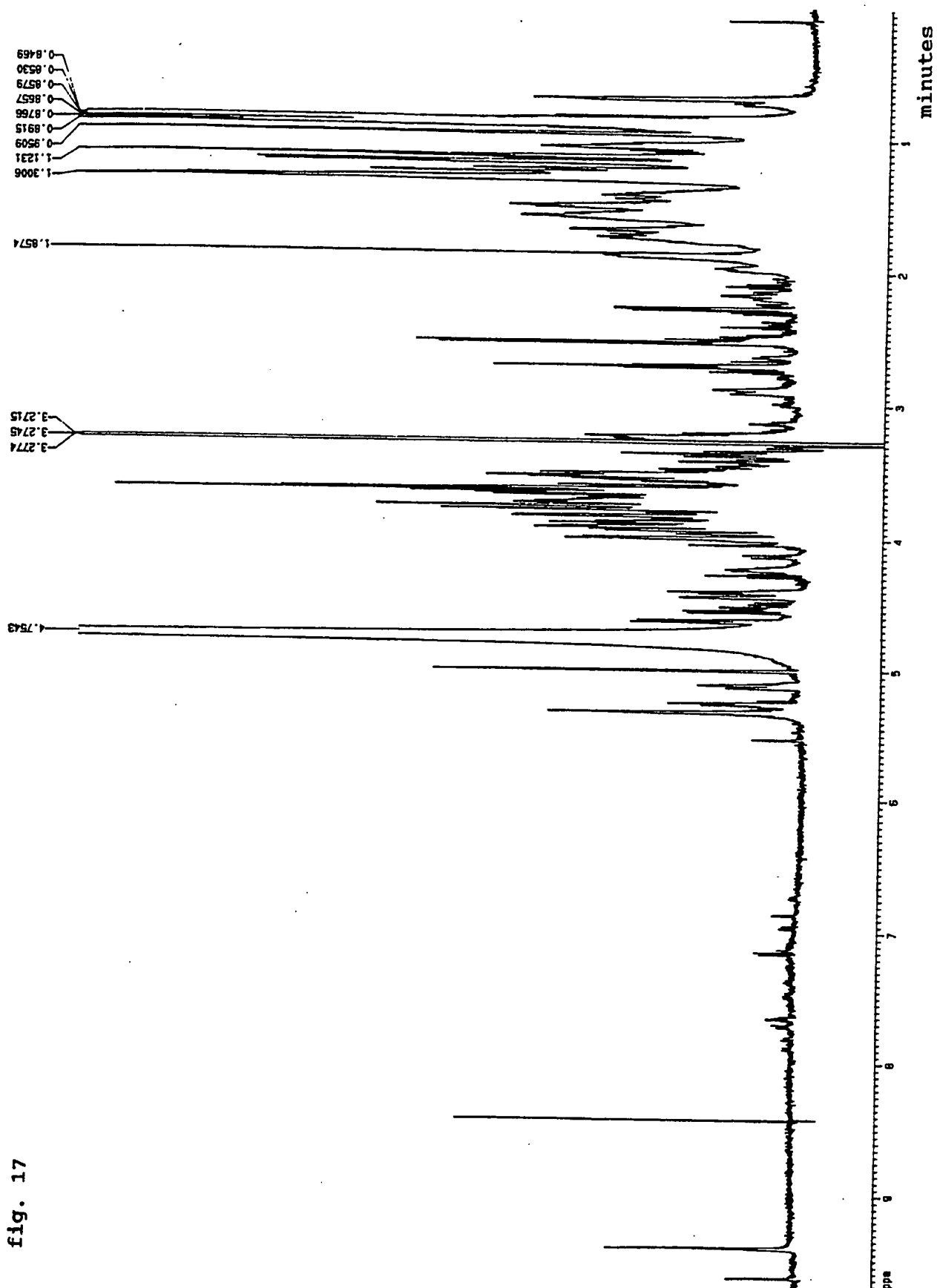


fig. 17

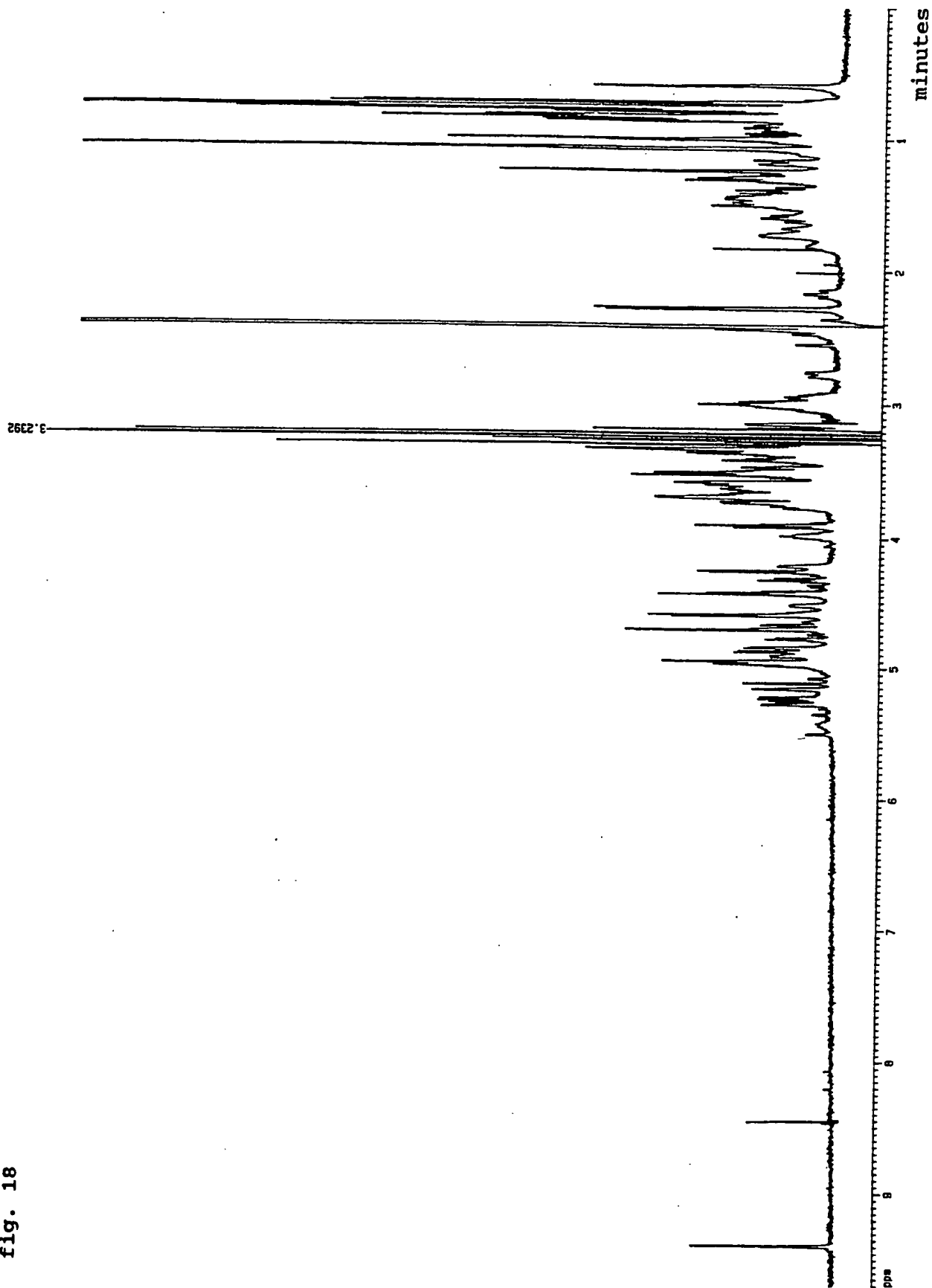
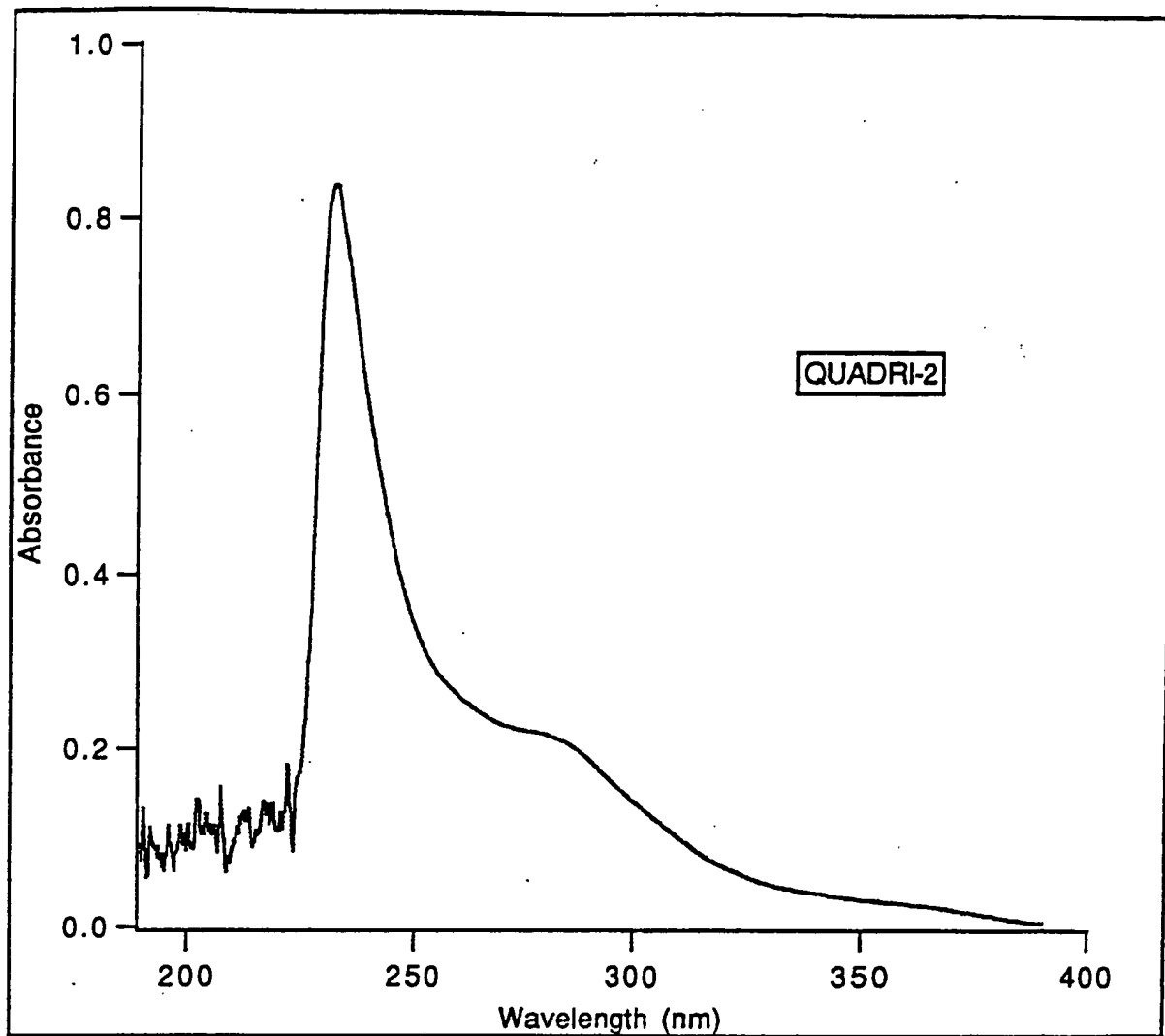
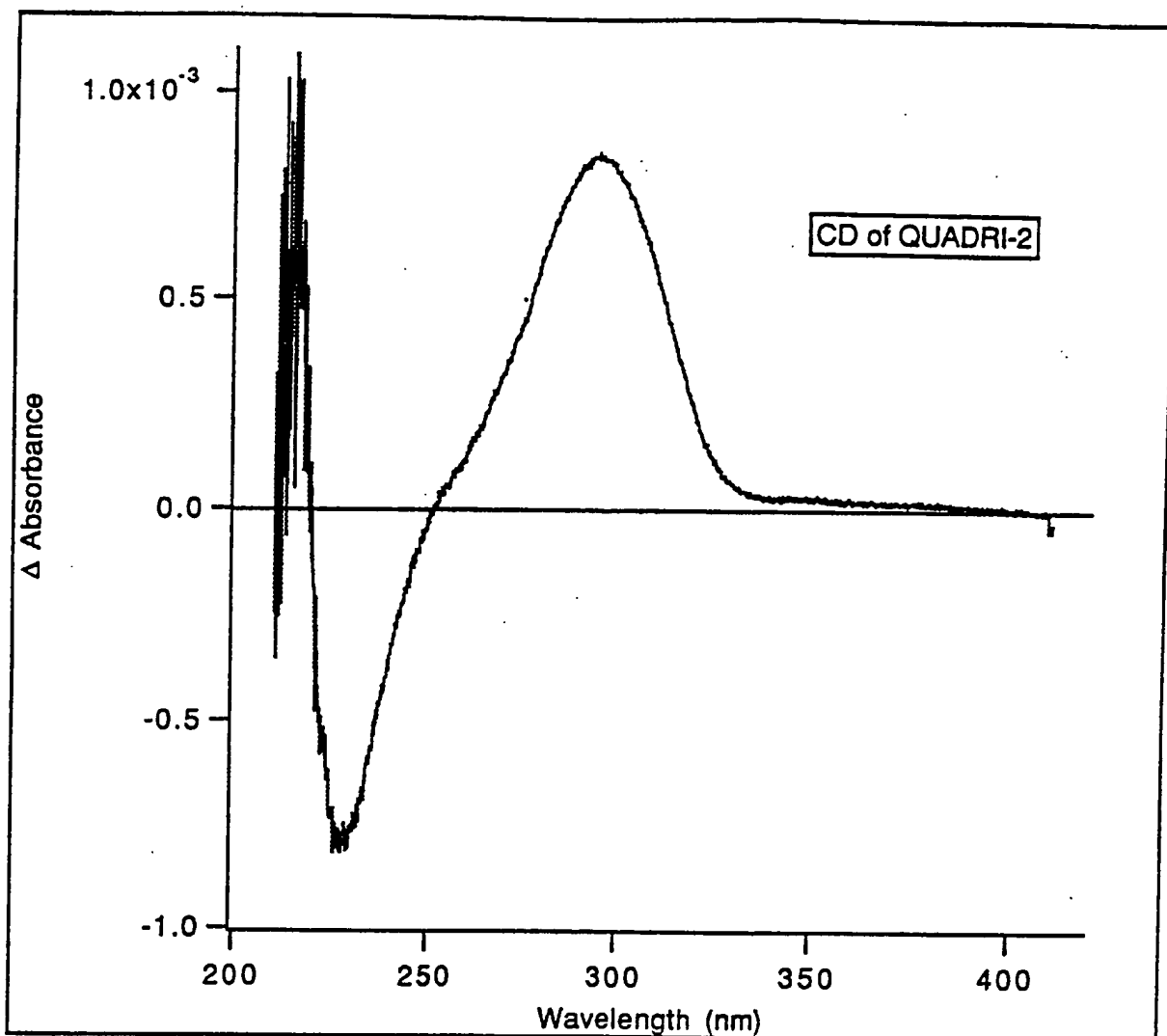


fig. 18





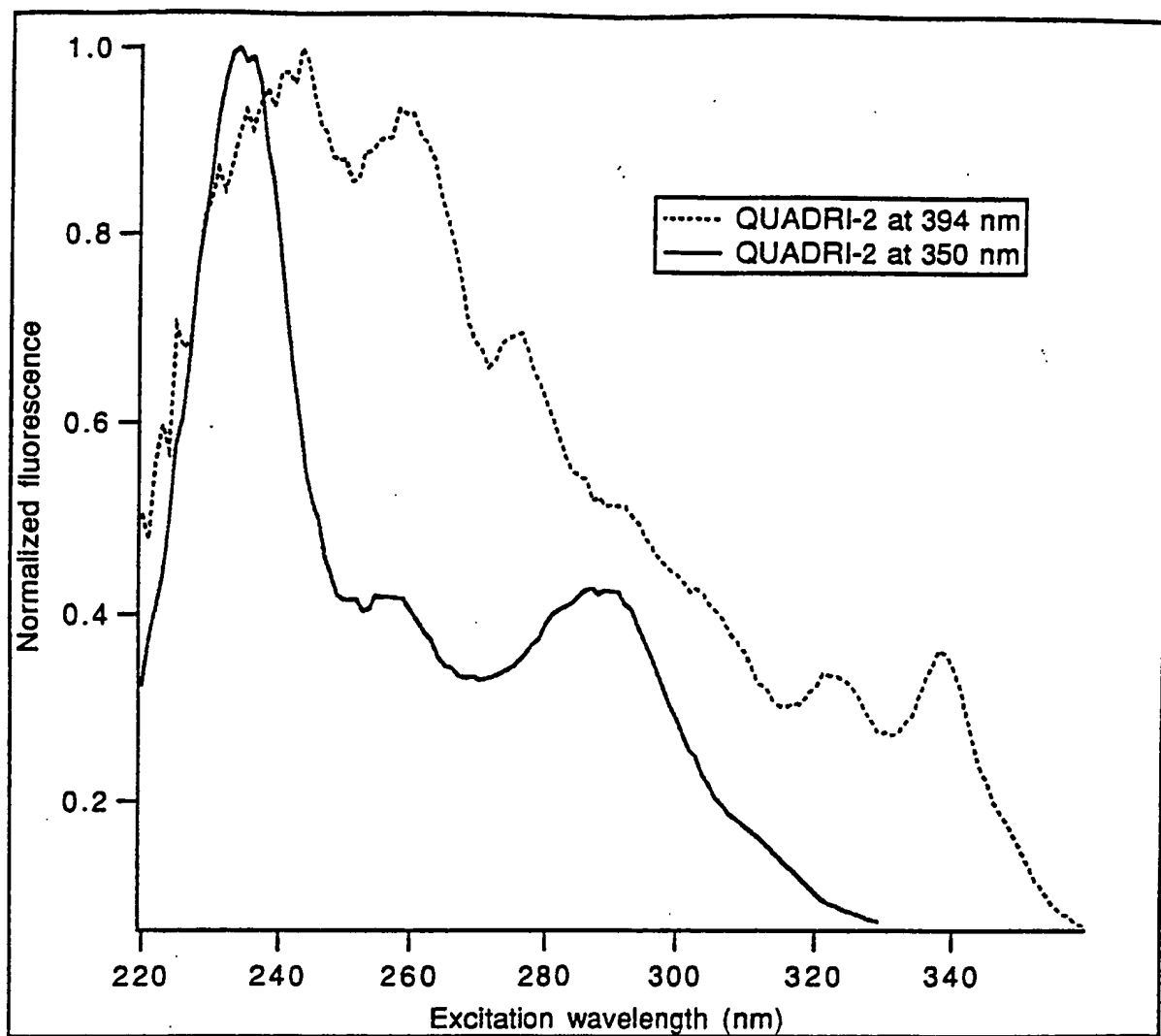
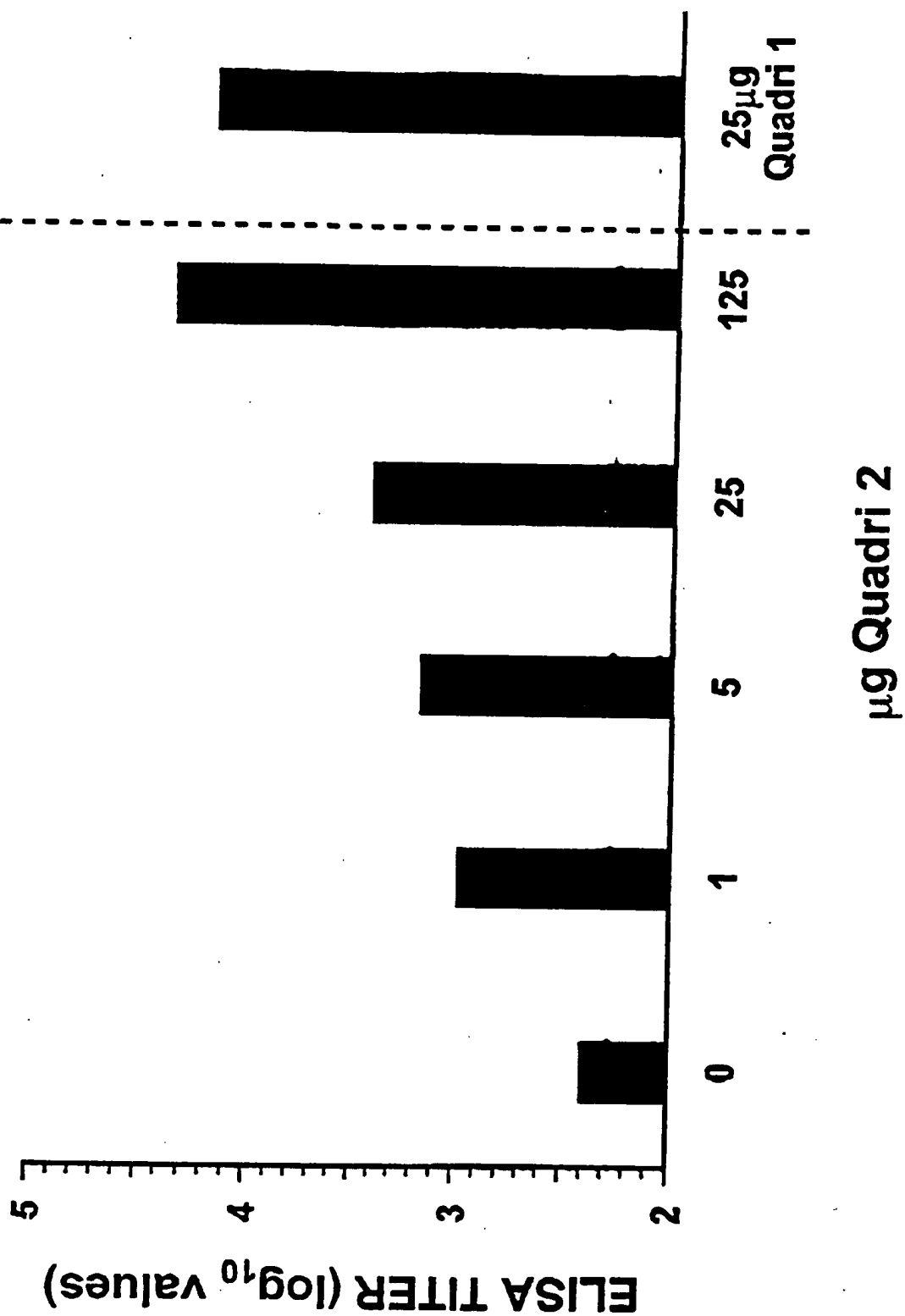
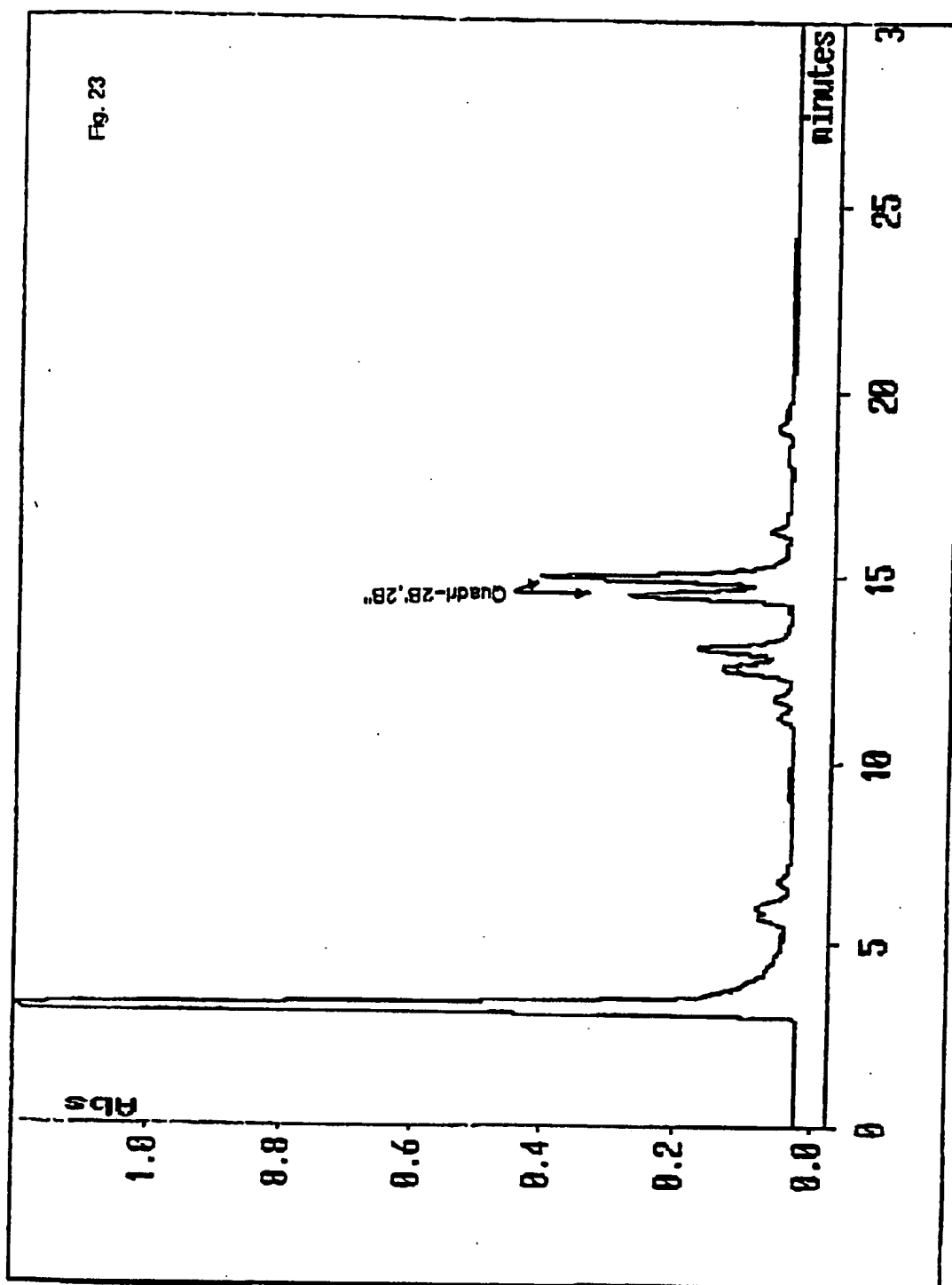
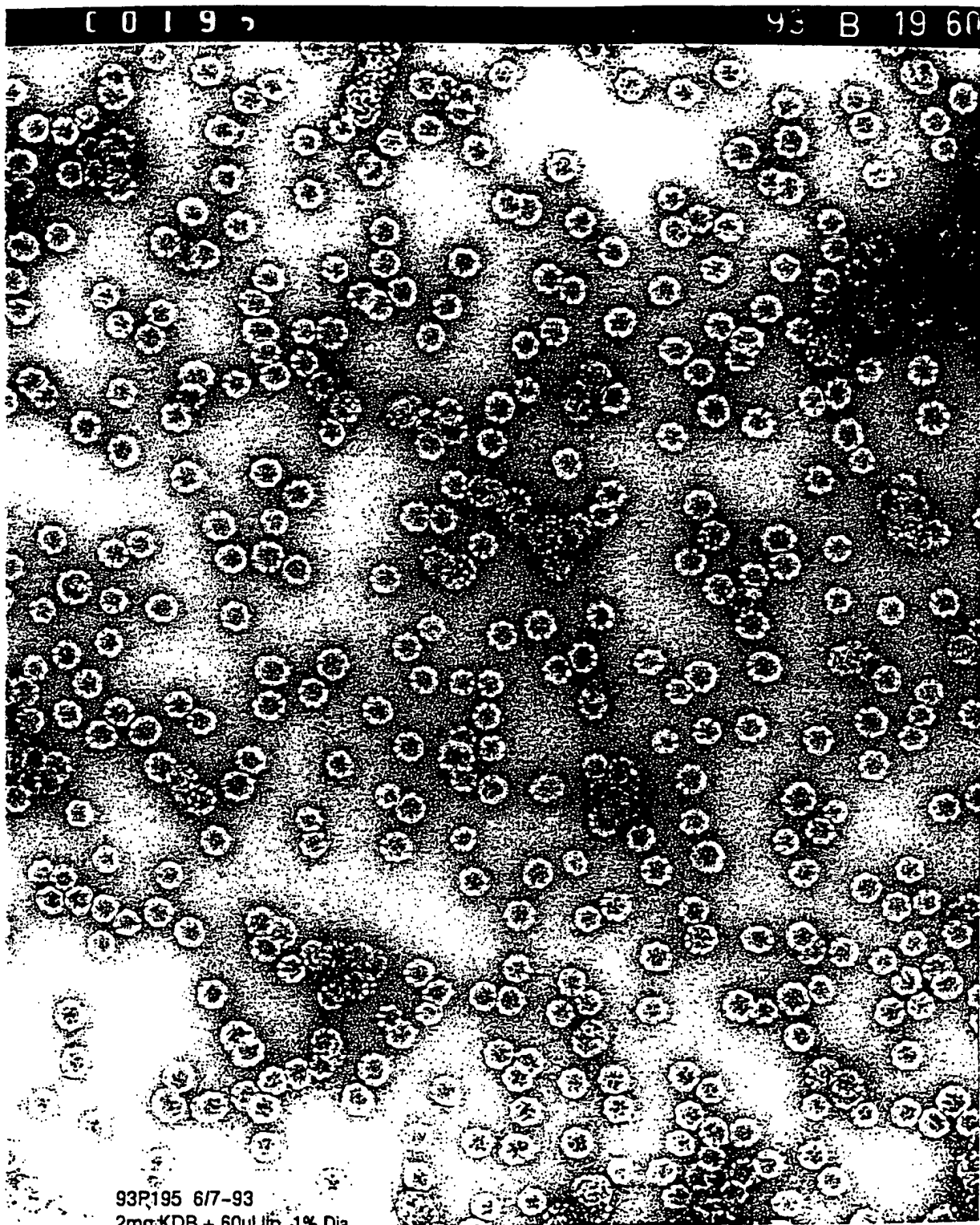


Fig. 22

Dose response experiment in guinea pigs
Antigen: 100 μ g Ovalbumin







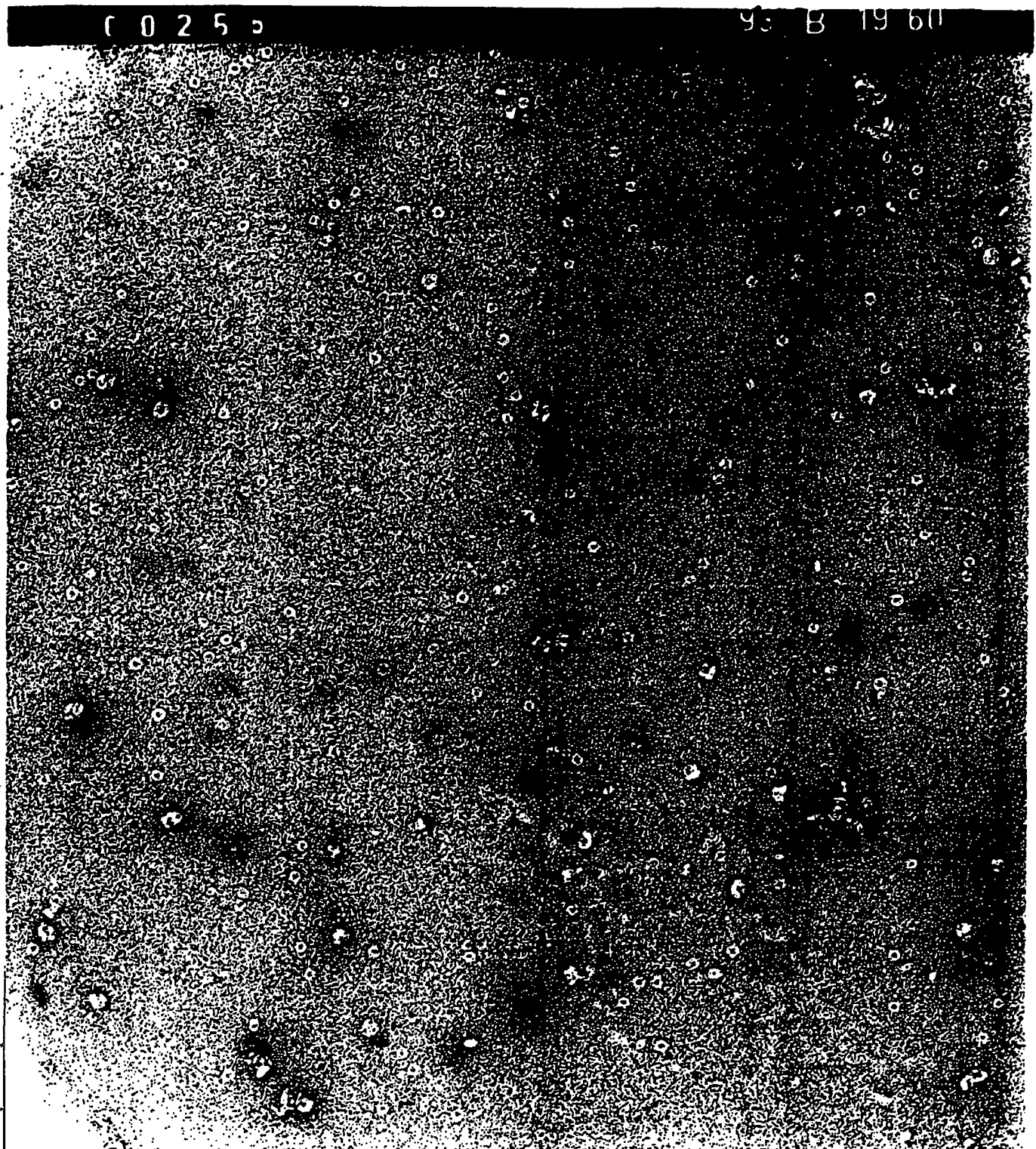
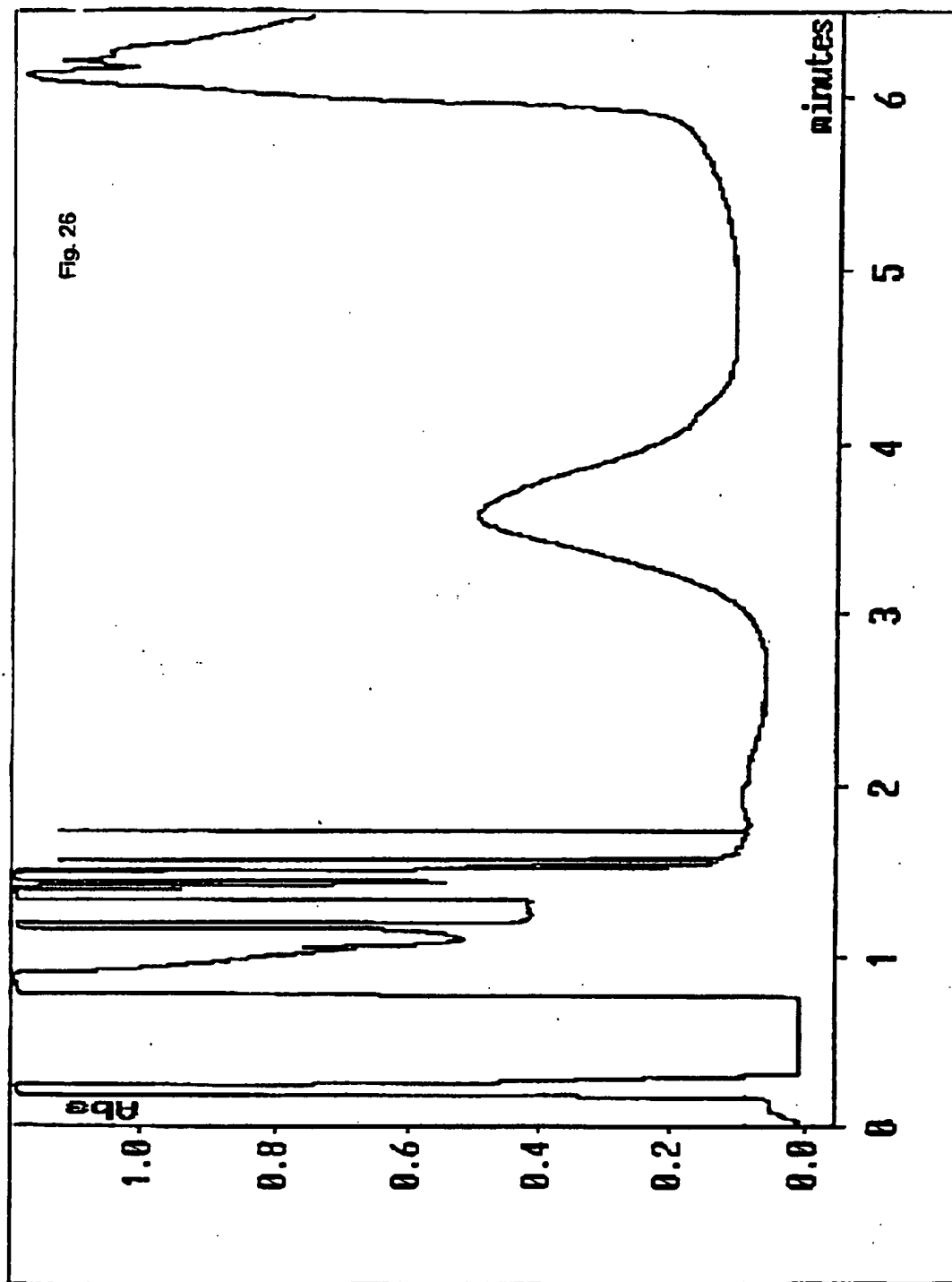
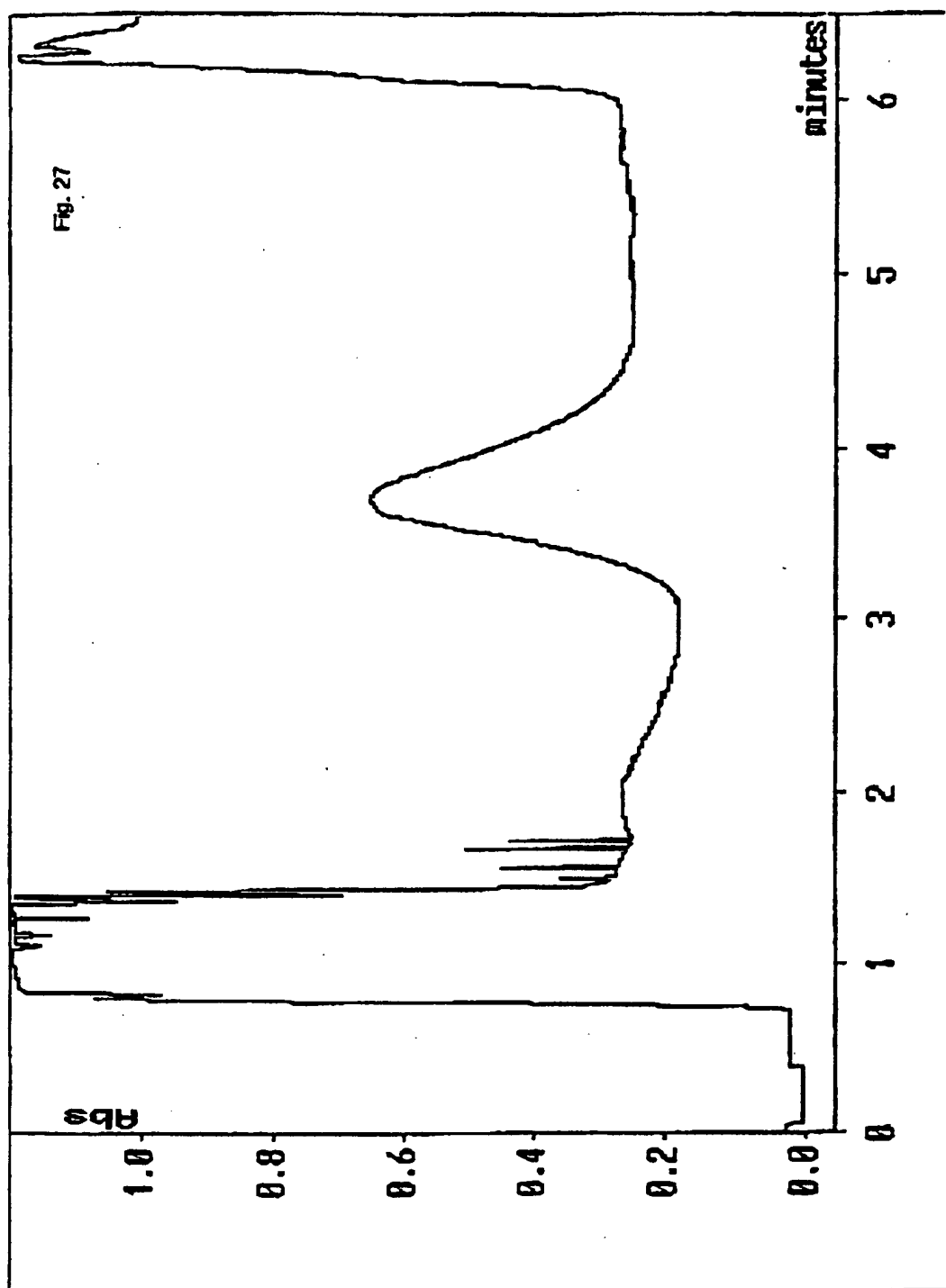


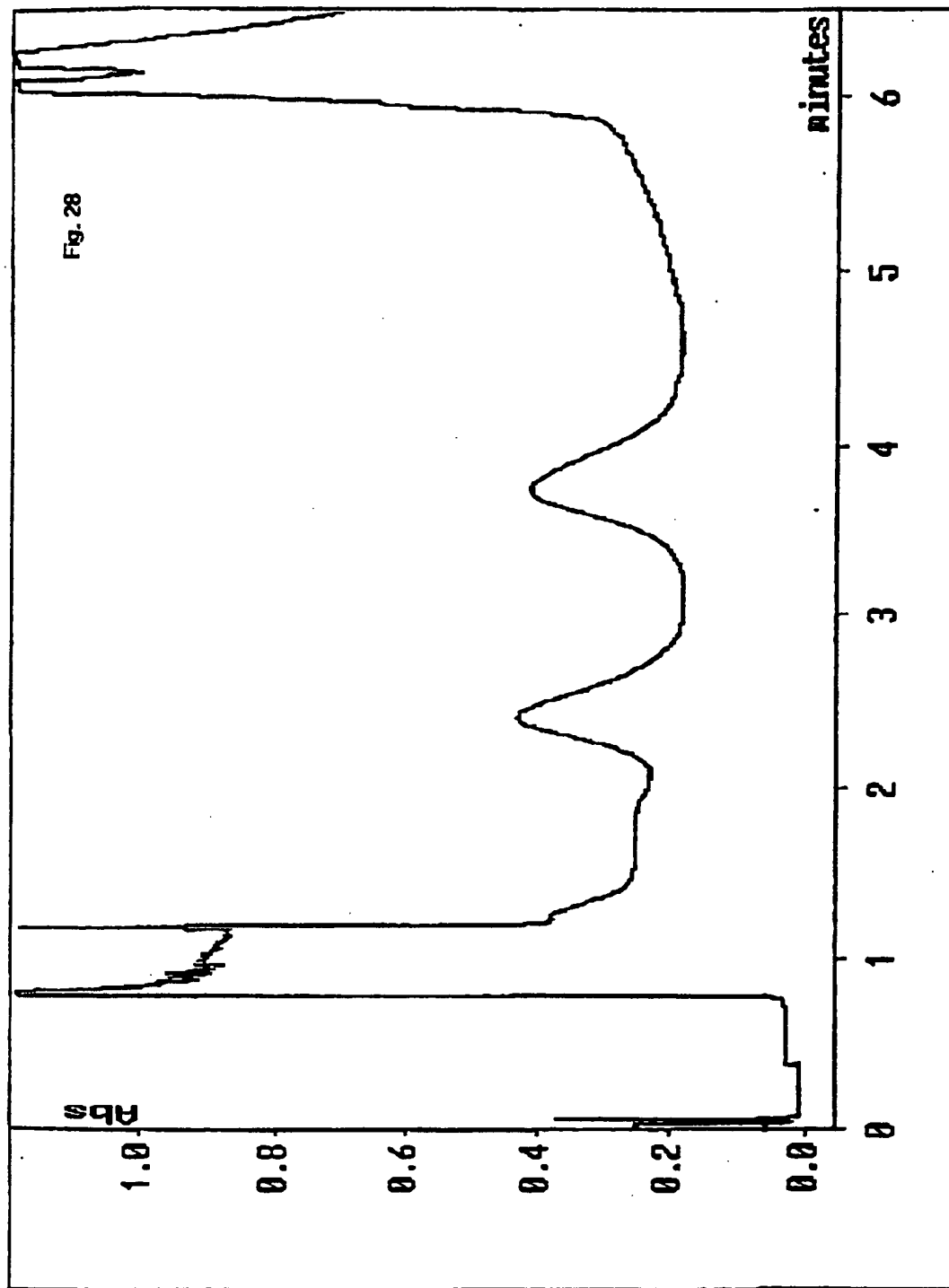
Fig. 25

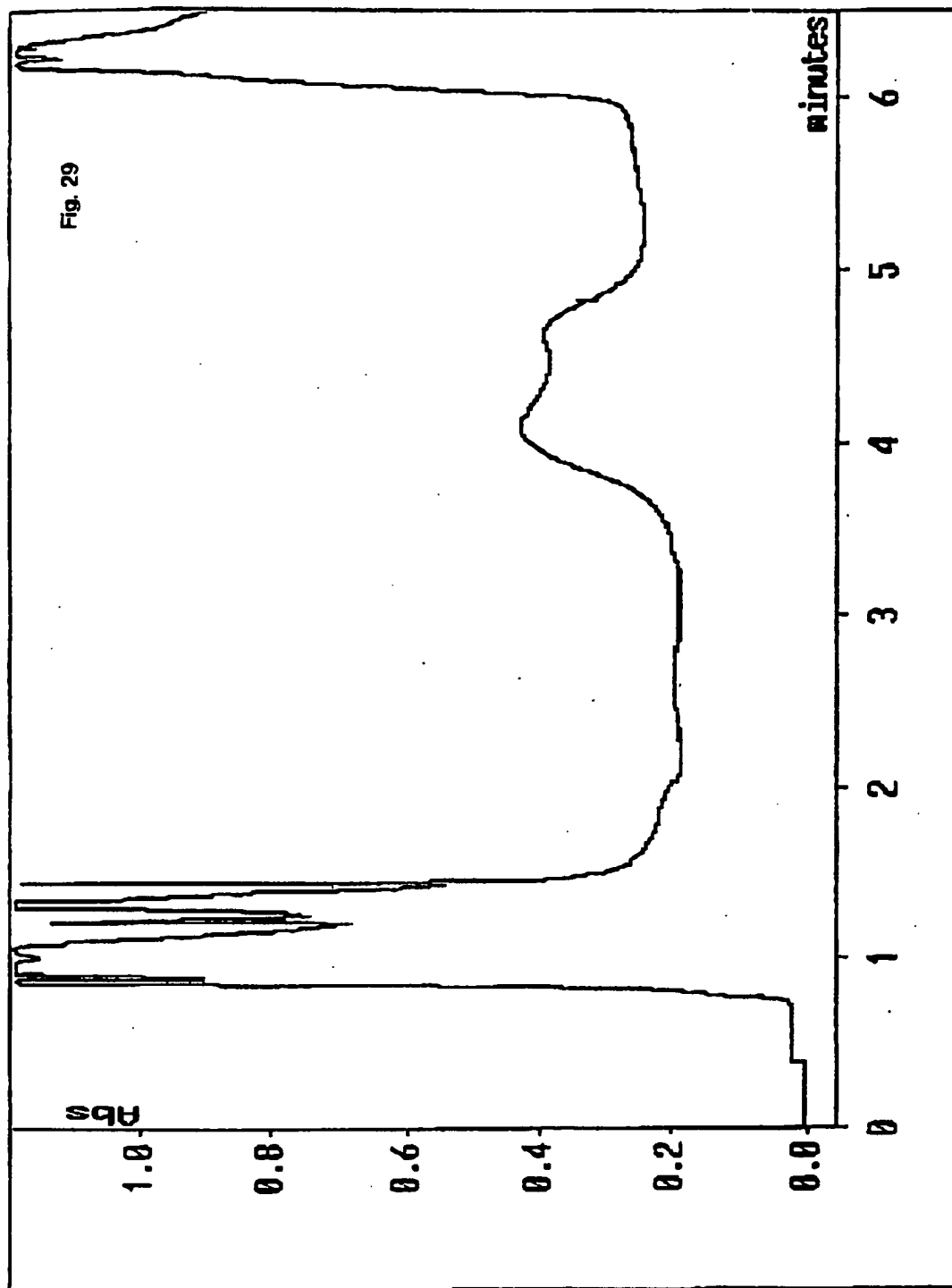


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INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/NL 94/00238

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07J63/00 C07H15/256 A61K31/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07J C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,90 03184 (B. MOREIN ET AL) 5 April 1990 cited in the application see example 6 ---	1-29
A	WO,A,92 06710 (DE STAAT DER NEDERLANDEN) 30 April 1992 cited in the application see the whole document ---	1-29
A	WO,A,93 05789 (CAMBRIDGE BIOTECH CORPORATION) 1 April 1993 cited in the application see the whole document ---	1-29
-/--		



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

25 January 1995

Date of mailing of the international search report

0 2. 02. 95

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INTERNATIONAL SEARCH REPORT

Int. Patent Application No
PCT/NL 94/00238

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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